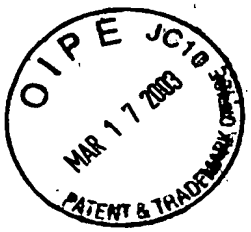

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section74(1) and (4) of the Deregulation and Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the international application filed on 09 AUGUST 2000 under the Patent Cooperation Treaty at the UK Receiving Office. The application was allocated the number PCT/GB00/03056

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or the inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed K E Taylor

Date: 27 January 2003

Home.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No PCT/GB 00/03056

(09.08.00)

International Filing Date 09 AUGUST 2000

United Kingdom Patent Office
PCT International Application

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* P/7130WORATM

Box No. I TITLE OF INVENTION

Loading Method

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

Gendel Limited
University of Ulster
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County Londonderry
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United Kingdom

✓

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: United Kingdom

State *(i.e. country)* of residence: United Kingdom

This person is applicant for the purposes of: ☐ all designated States ☑ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

✓ FADLON, Emma
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Added Rolls.

This person is:

☐ applicant only

☑ applicant and inventor

☐ inventor only *(if this check-box is marked, do not fill in below)*

State *(i.e. country)* of nationality: United Kingdom

State *(i.e. country)* of residence: United Kingdom

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☑ the United States of America only ☐ the States indicated in the Supplemental Box

☑ Further applicants and/or (further) inventors are indicated on a continuation sheet

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☑ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

PCT/GB 00/03056

Continuation of Box No. III FURTHER APPLICANT (S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

McHALE, Anthony Patrick
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United Kingdom

This person is:
- ☐ applicant only
- ☑ applicant and inventor
- ☐ inventor only *(if this check-box is marked, do not fill in below)*

State *(that is, country)* of nationality: ~~United Kingdom~~ IE.

State *(that is, country)* of residence: United Kingdom

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☑ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CRAIG, Roger
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Sandbach
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United Kingdom

This person is:
- ☐ applicant only
- ☑ applicant and inventor
- ☐ inventor only *(if this check-box is marked, do not fill in below)*

State *(that is, country)* of nationality: United Kingdom

State *(that is, country)* of residence: United Kingdom

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☑ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(if this check-box is marked, do not fill in below)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(if this check-box is marked, do not fill in below)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☑ **AP** ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☑ **EA** Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☑ **EP** European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☑ **OA** OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, please specify on dotted line)*

.....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☑ **AE** United Arab Emirates
- ☑ **AL** Albania
- ☑ **AM** Armenia
- ☑ **AT** Austria
- ☑ **AU** Australia
- ☑ **AZ** Azerbaijan
- ☑ **BA** Bosnia and Herzegovina
- ☑ **BB** Barbados
- ☑ **BG** Bulgaria
- ☑ **BR** Brazil
- ☑ **BY** Belarus
- ☑ **CA** Canada
- ☑ **CH AND LI** Switzerland and Liechtenstein
- ☑ **CN** China
- ☑ **CR** Costa Rica
- ☑ **CU** Cuba
- ☑ **CZ** Czech Republic
- ☑ **DE** Germany
- ☑ **DK** Denmark
- ☑ **DM** Dominica
- ☑ **EE** Estonia
- ☑ **ES** Spain
- ☑ **FI** Finland
- ☑ **GB** United Kingdom
- ☑ **GD** Grenada
- ☑ **GE** Georgia
- ☑ **GH** Ghana
- ☑ **GM** Gambia
- ☑ **HR** Croatia
- ☑ **HU** Hungary
- ☑ **ID** Indonesia
- ☑ **IL** Israel
- ☑ **IN** India
- ☑ **IS** Iceland
- ☑ **JP** Japan
- ☑ **KE** Kenya
- ☑ **KG** Kyrgyzstan
- ☑ **KP** Democratic People's Republic of Korea
- ☑ **KR** Republic of Korea
- ☑ **KZ** Kazakhstan
- ☑ **LC** Saint Lucia
- ☑ **LK** Sri Lanka
- ☑ **LR** Liberia
- ☑ **LS** Lesotho
- ☑ **LT** Lithuania
- ☑ **LU** Luxembourg
- ☑ **LV** Latvia
- ☑ **MA** Morocco
- ☑ **MD** Republic of Moldova
- ☑ **MG** Madagascar
- ☑ **MK** The former Yugoslav Republic of Macedonia
- ☑ **MN** Mongolia
- ☑ **MW** Malawi
- ☑ **MX** Mexico
- ☑ **NO** Norway
- ☑ **NZ** New Zealand
- ☑ **PL** Poland
- ☑ **PT** Portugal
- ☑ **RO** Romania
- ☑ **RU** Russian Federation
- ☑ **SD** Sudan
- ☑ **SE** Sweden
- ☑ **SG** Singapore
- ☑ **SI** Slovenia
- ☑ **SK** Slovakia
- ☑ **SL** Sierra Leone
- ☑ **TJ** Tajikistan
- ☑ **TM** Turkmenistan
- ☑ **TR** Turkey
- ☑ **TT** Trinidad and Tobago
- ☑ **TZ** United Republic of Tanzania
- ☑ **UA** Ukraine
- ☑ **UG** Uganda
- ☑ **US** United States of America
- ☑ **UZ** Uzbekistan
- ☑ **VN** Viet Nam
- ☑ **YU** Yugoslavia
- ☑ **ZA** South Africa
- ☑ **ZW** Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after the issuance of this sheet:

- ☑ **DZ** Algeria
- ☑ **AG** Antigua and Barbuda
- ☑ **MZ** Mozambique
- ☑ **BZ** Belize

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

Use this box in the following cases:

1. If, in any of the Boxes, the space is insufficient to furnish all the information:

in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;

in particular:

(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: — in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below;

(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: — in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;

(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: — in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;

(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: — in such case, write "Continuation of Box No. IV and indicate for each further agent the same type of information as required in Box No. IV;

(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part": — in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

(vi) if there are more than three earlier applications whose priority is claimed: — in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.

2. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty:

in such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.

Continuation of Box No. IV
COTTER, Ivan John
PILCH, Adam John Michael
CRISP, David Norman
ROBINSON, Nigel Alexander Julian
HARRIS, Ian Richard
HARDING, Charles Thomas
TURNER, James Arthur
MALLALIEU, Catherine Louise
PRATT, Richard Wilson
PRICE, Paul Anthony King
HOLMES, Miles
HORNER, David Richard
MASCHIO, Antonio
NACHSHEN, Neil
POTTER, Julian
HAINES, Miles John
MATHER, Belinda Jane
BODEN, Keith McMurray
DEVILE, Jonathan Mark

Box No. VI PRIORITY CLAIM

☐ Further priority claims are indicated in the Supplemental Box

	Filing Date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application: * regional Office	international application: receiving Office
item (1)	8 Feb 2000 8/2/2000	0002856.3 ✓	GB		
item (2)	11 Feb 2000 11/2/2000	60/181,796	US		
item (3)	24 Jul 2000 24/7/2000	PCT/GB00/02848 ✓	PCT		

☑ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s) : (1) and (3)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA / EPO

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number: Country (or regional Office):

Box No. VII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	:	5 ✓
description (excluding sequence listing part)	:	51 ✓
claims	:	6 ✓
abstract	:	1 ✓
drawings	:	12 ✓
sequence listing part of description	:	
Total number of sheets	:	75 ✓

This international application is accompanied by the item(s) marked below:

1. ☑ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority documents(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☑ other *(specify)*: Letter

Figure of the drawings which should accompany the abstract: 1

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

Dr. Miles Haines

HAINES, Miles

For receiving Office use only

1. Date of actual receipt of the purported international application: 9 AUGUST 2000 (09.08.00)
2. Drawings: ☑ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

LOADING METHOD

FIELD OF THE INVENTION

The present invention relates to a method for loading a red blood cell with an agent, which cell may be sensitised to assist in agent release.

BACKGROUND TO THE INVENTION

The delivery of therapeutic agents to specific tissues is desirable typically to ensure that a sufficiently high dose of a given agent is delivered to a selected tissue. Moreover, it is often the case that the therapeutic agent, although advantageously having beneficial therapeutic effects on the diseased tissue, may have undesirable side effects on tissues that are not diseased. For example, in the treatment of certain types of disorders, such as cancer, it is necessary to use a high enough dose of a drug to kill the cancer cells without killing an unacceptable high number of normal cells. Thus, one of the major challenges of disease treatment is to identify ways of exploiting cellular drug delivery vehicles to incorporate and to selectively release agents at a desired target site.

It has been suggested that red blood cells may be exploited as active agent/drug delivery vehicles (DeLoach & Sprandel 1985, *Bibliotheca Haematologica*; Publ. Karger, Munich) as it is possible to incorporate agents into human red blood cells using a variety of techniques. An example of such a technique is the exploitation of osmotic shock and modifications thereof such as hypotonic shock and subsequent recovery of isotonicity and reverse hypotonic dialysis (Luque & Pinilla, 1993, *Ind. Farmac.* 8, 53-59).

An alternative method for loading drugs and active agents into red blood cells is electroporation. Using this process, the agent of interest is mixed with the live red blood cells in a buffer medium and short pulses of high electric fields are applied. The red blood cell membranes are transiently made porous and the agents of interest enter the cells. The electroporation process is advantageous as very high loading indices can be achieved within a very short time period (Flynn *et al.*, 1994, *Cancer Letts.*, 82, 225-229).

When packaging/carrier/delivery systems such as red blood cells are used as *in vivo* delivery systems, they suffer from the drawback that the delivery function is dependent upon both an accumulation of the red blood cells and a breakdown of the red blood cell membrane in or at the relevant tissue/site. As a result, attempts have been made to incorporate sensitising agents into cell carriers in order to facilitate both the accumulation and/or release of an agent of interest at a target site.

By way of example, our UK Patent Applications 9816583.0 and 9826676.0 (incorporated by reference) relate *inter alia* to the incorporation of a dye compound, such as a porphyrin, which renders a loaded red blood cell susceptible to laser light treatment at a target site. This phenomenon, known as photodynamic activation, is exploited in order to achieve accumulation of the carrier vehicle at the relevant site and to achieve load release at that site.

Alternative energy sources have been investigated as tools for inducing payload release from loaded and sensitised cells. By way of example, ultrasound has been investigated as an alternative to light induced photodynamic activation as it has a broader degree of focus and it penetrates more deeply into the body. However, although ultrasound has also been applied to effect red blood cell lysis *in vitro*, its use has been limited in that its effect is only significant at lower cell concentrations (1-6x10^6 cells) (Brayman *et al.*, 1996, *Ultrasound in Med & Biol.*, 22: 497-514). Moreover, ultrasound is non-specific in its effects, resulting in lysis of both loaded and endogenous red blood cells.

Recently, it has been found that certain dye compounds, in particular porphyrins, can achieve a cytopathogenic effect when the disease site is subjected to ultrasound. This technique is referred to as sonodynamic therapy and is discussed in WO98/52609. WO98/52609 teaches that ultrasound may be useful in treating disease but only when it is combined with an effective amount of an ultrasound-susceptibility modification agent such as a porphyrin.

In our U.K. patent application No. 9917416.1, incorporated by reference, we have shown that treatment of red blood cells with an electric field increases their sensitivity to ultrasound mediated disruption. Consequently, efficient unloading of therapeutic agents carried by red blood cells at a site of interest can be achieved at lower exposures of ultrasound, reducing possible damage to normal red blood cells.

SUMMARY OF THE INVENTION

We have now further shown that if the red blood cells are pre-sensitised prior to a dialysis loading step, close to 100% efficiency in the loading step can be achieved. A first aspect of the invention relates to this finding.

However we have also found that the dialysis loading step reduces the sensitivity of the loaded cells to ultrasound. This reduction in sensitivity can be reversed by subjecting the cells to an additional sensitisation step regardless of whether the additional step is performed before or after loading. Using this pre-sensitisation/sensitisation procedure, red blood cells can be produced that have both excellent loading characteristics and ultrasound sensitivity. Consequently, highly efficient unloading of therapeutic agents carried by red blood cells at a site of interest can be achieved at low exposures of ultrasound. This represents a considerable improvement over prior art methods. The present invention therefore provides an improved method for selectively releasing an agent from a loaded red blood cell at a target site, as further aspects.

According to a first aspect of the present invention, we provide a method of producing a red blood cell suitable for delivery of an agent to a vertebrate, the method comprising: (a) providing a red blood cell; (b) pre-sensitising the red blood cell; and (c) loading the red blood cell with an agent.

Preferably, the amount of agent that is loaded into a pre-sensitised red blood cell is higher than the amount loaded into a red blood cell which is not pre-sensitised.

More preferably, the method further comprises the step of electrosensitising the cell to render it more susceptible to disruption by exposure to a stimulus, the loading step and the electrosensitisation step being performed in any order.

There is provided, according to a second aspect of the present invention, a method for selectively releasing an agent from a red blood cell comprising the steps of: (a) pre-sensitising a red blood cell; (b) loading the cell with an agent; (c) electrosensitising the cell; and (d) causing the agent to be released from the sensitised cell by applying ultrasound at a frequency and energy sufficient to cause disruption of the sensitised cell but insufficient to cause disruption of un-sensitised red blood cells, in which steps (b) and (c) can be performed in any order.

We provide, according to a third aspect of the present invention, a method for delivering an agent to a target site in a vertebrate, the method comprising a method according to the second aspect of the invention, with the further step of introducing the cell into a vertebrate between steps (c) and (d).

The red blood cell may be PEGylated prior to being introduced into the vertebrate. Preferably, the vertebrate is a mammal. In each of the above-mentioned methods, either or both of the pre-sensitising step and the electrosensitising step may be an *in vitro* or *ex-vivo* procedure.

The pre-sensitising may comprise a step of applying an electric field to the red blood cell. Alternatively, or in addition, the pre-sensitising may comprise a step of applying ultrasound to the red blood cell.

In a preferred embodiment of the invention, the red blood cell is loaded with the agent by hypotonic dialysis.

Preferably, the electrosensitising comprises the step of applying an electric field to the red blood cell. More preferably, the electric field is from about 0.1 kV/cm to about 10

kV/cm under *in vitro* conditions. Most preferably, the electric field is applied for between 1 μs and 100 ms.

Where the red blood cell is subject to electrosensitisation, the electrosensitisation of the red blood cell may be performed after the loading of the agent. Alternatively, the electrosensitisation of the red blood cell is performed before the loading of the agent.

The ultrasound may be selected from the group consisting of diagnostic ultrasound, therapeutic ultrasound and a combination of diagnostic and therapeutic ultrasound. Preferably, the applied ultrasound energy source is at a power level of from about 0.05 W/cm^2 to about 100 W/cm^2.

As a fourth aspect of the present invention, there is provided a red blood cell delivery vector which has been pre-sensitised such that it is capable of being loaded with a larger amount of agent than a red blood cell which has not been pre-sensitised. Preferably, the red blood cell delivery vector has been pre-sensitised by exposure to an electric field and/or ultrasound. More preferably, the red blood cell delivery vector is sensitised to render it more susceptible to disruption by exposure to a stimulus. Most preferably, the red blood cell delivery vector is loaded with an agent to be delivered.

The agent is preferably selected from a group consisting of a protein, a polypeptide, a peptide, a nucleic acid, a peptide nucleic acid (PNA), a virus, a virus-like particle, a nucleotide, a ribonucleotide, a deoxyribonucleotide, a modified deoxyribonucleotide, a heteroduplex, a nanoparticle, a synthetic analogue of a nucleotide, a synthetic analogue of a ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analogue, a modified amino acid, a modified amino acid analogue, a steroid, a proteoglycan, a lipid, a fatty acid, an oligosaccharide, a glycoprotein, a carbohydrate, and mixtures, fusions, combinations or conjugates of the above. The agent may be conjugated to, fused to, mixed with or combined with an imaging agent.

We provide, according to a fifth aspect of the present invention, a red blood cell delivery vector obtainable by a method comprising: (a) pre-sensitising a red blood cell by

electrosensitising the cell; (b) loading the cell with an agent; and (c) electrosensitising the cell, in which steps (b) and (c) can be performed in any order.

The present invention, in a sixth aspect, provides the use of an electric field and/or ultrasound to increase the efficiency of loading of an agent into a red blood cell.

Preferably, the efficiency of loading of a pre-sensitised cell is 50% or greater, more preferably, 60% or greater, even more preferably, 70% or greater, yet more preferably, 80% or greater. In highly preferred embodiments of the invention, the loading efficiency of a cell pre-sensitised according to our invention is 90% or greater, preferably 95% or 100%. Loading efficiency as used here refers to the percentage of cells which have taken up agent compared with the starting population. Various means may be used for assessing loading efficiency, for example, FACS analysis as described here.

In a seventh aspect of the present invention, there is provided a method of pre-sensitising a red blood cell with an electric field and/or ultrasound such that the amount of agent that is capable of being loaded into the pre-sensitised red blood cell is higher than that which is capable of being loaded into a red blood cell which is not pre-sensitised.

According to an eighth aspect of the present invention, we provide a kit comprising a red blood cell made by a method according to the first, second or third aspect of the invention or a red blood cell delivery vector according to the fourth or fifth aspect of the invention, packaging materials therefor and instructions for use.

We provide, according to a ninth aspect of the invention, a kit comprising a red blood cell, an agent, packaging materials therefor and instructions for use in a method comprising the steps of: (a) pre-sensitising a red blood cell; (b) loading the cell with an agent; (c) electrosensitising the cell; and (d) causing the agent to be released from the sensitised cell by applying ultrasound at a frequency and energy sufficient to cause disruption of the sensitised cell but insufficient to cause disruption of un-sensitised red blood cells, in which steps (b) and (c) can be performed in any order.

There is provided, according to a tenth aspect of the invention, a kit comprising a pre-sensitised red blood cell which is loaded with an agent, packaging materials therefor and instructions for use in a method comprising the steps of: (a) electrosensitising the cell; and (b) causing the agent to be released from the sensitised cell by applying ultrasound at a frequency and energy to cause disruption of the sensitised cell but insufficient to cause disruption of unsensitised red blood cells.

As an eleventh aspect of the invention, we provide a kit comprising a red blood cell delivery vector according to the fifth aspect of the invention, packaging materials therefor and instructions for use comprising the step of causing the agent to be released from the red blood cell delivery vector by applying ultrasound at a frequency and energy to cause disruption of the red blood cell delivery vector but insufficient to cause disruption of un-sensitised red blood cells.

Preferably, the kit further comprises polyethylene glycol. More preferably, the kit further comprises a liquid selected from the group consisting of a buffer, diluent or other excipient. The liquid may be selected from the group consisting of a saline buffer, a physiological buffer, serum and plasma.

We provide, according to a twelfth aspect of the invention, a pharmaceutical composition comprising a red blood cell made by a method according to the first, second or third aspect of the invention or a red blood cell delivery vector according to the fourth or fifth aspect of the invention, together with a pharmaceutically acceptable carrier or diluent.

There is further provided a method and/or a red blood cell delivery vector and/or a kit and/or a pharmaceutical composition substantially as described herein and with reference to the examples and figures.

Furthermore, we provide a device for producing a red blood cell delivery vector of the present invention which device comprises: (a) one or more flow cells and electrosensitisation means; (b) one or more dialysis systems; in which the flow cell is

linked to the dialysis system by connecting means capable of allowing the transfer of red blood cells from the flow cell to the dialysis system and vice versa.

Optionally, the device may comprise more than one flow cell, such as two flow cells. The device may also be connected to a collection device such as a blood bag. In a preferred embodiment, the device is also connected to a chromatography and/or filtration stage so that after the final sensitisation procedure, red blood cells are purified and/or the composition of buffer in which the cells are suspended altered.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows flow cytometry profiles obtained following analysis of human erythrocytes loaded with a fluorescently-labelled oligonucleotide. The X axis represents the fluorescence intensity exhibited by cells in each preparation and the Y-axis represents the number of counts detected by the flow cytometer at a given fluorescence intensity. The control (indicated by RBC) represents the profile exhibited by human erythrocytes which had been placed in contact with the oligonucleotide. Movement of the electroporated peak to the right is indicative of loading of oligonucleotide into the preparation.

Figure 1B shows flow cytometry profiles obtained following analysis of cell preparations loaded with oligonucleotide using conventional dialysis (– • –) and electropulsing combined with dialysis (Gendel dialysis, —). Control samples consist of human erythrocytes alone (RBC) and erythrocytes together with oligonucleotide without any further treatment (oligo no dialysis). The X-axis represents the fluorescence intensity and the Y-axis represents the number of counts detected at any given fluorescence intensity.

Figure 2A shows flow cytometry profiles obtained following analysis of human erythroyctes loaded with FITC-labelled antibody by electroporation (—) (exponential decay pulsing). The control sample (RBC+ab) represents the profile exhibited by cells exposed to antibody in the absence of an electroporating pulse. The X-axis represents the

fluorescence intensity and the Y-axis represents the number of counts detected at any given fluorescence intensity.

Figure 2B shows flow cytometry profiles obtained following analysis of human erythrocytes loaded with FITC-labelled antibody by electroporation (—) (square wave pulse). The control sample (RBC+ab) represents the profile exhibited by cells exposed to antibody in the absence of an electroporating pulse. The X-axis represents the fluorescence intensity and the Y-axis represents the number of counts detected at any given fluorescence intensity.

Figure 3 shows flow cytometry profiles obtained following analysis of human erythroyctes loaded with FITC-labelled antibody by conventional published dialysis (—) and by the dialysis process as described in Example 1 (—). The X-axis represents the fluorescence intensity and the Y-axis represents the number of counts detected at any given fluorescence intensity.

Figure 4 shows flow cytometry profiles obtained following analysis of human erythroyctes loaded with FITC-labelled antibody using buffer A (**A**) and buffer B (**B**). The peak on the left represents the control population prior to loading and that on the right represents the profile obtained following loading. The X-axis represents the fluorescence intensity and the Y-axis represents the number of counts detected at any given fluorescence intensity.

Figure 5 shows ultrasound-mediated release of anti von Willebrand factor antibody from sensitised human erythrocytes in perfused rat kidney.

Figure 6 shows the stability of cellular integrity (cell numbers) and ultrasound sensitivity during storage at 4°C. Cells were loaded with FITC labelled antibody using a process comprising pre-sensitisation/hypotonic dialysis/electrosensitisation (ES-HD-ES) and at the indicated times cell numbers (▲) were determined by direct counting. The percentage of cells that lysed following exposure to ultrasound was also determined (◆) for each sample. The X-axis represents the time in days, the left Y-axis represents the

percentage of cells remaining intact and the right Y-axis represents the percentage lysis observed following exposure to ultrasound.

Figure 7 shows flow cytometry profiles obtained following analysis of payload retention in samples stored at time zero and 30 days at 4°C. The peak on the right in the 30 day old sample exhibited a similar fluorescence intensity to that analysed at time zero indicating that the payload was retained throughout the storage period. The X-axis represents the fluorescence intensity and the Y-axis represents the number of counts detected at any given fluorescence intensity.

Figure 8 is a graph showing the ultrasound-mediated release of antibody from the erythrocyte vehicle. X-axis: power density (W/cm^2), left hand Y-axis: μg of anti-vWF released (per $7x10^7$ cells treated by ultrasound); right hand Y-axis: percentage of cells lysed by ultrasound. Filled squares represent μg antibody, loaded cells; filled triangles represent μg antibody, control cells; open squares represent % lysis, loaded cells; open triangles represent % lysis, control cells.

Figure 9 is a graph showing ultrasound mediated release of β-galactosidase from the erythrocyte vehicle. X-axis: power density (W/cm^2), left hand Y-axis: percentage lysis; right hand Y-axis: % relative enzyme release. Filled squares represent control lysis, filled triangles represent sample lysis and filled diamonds represent sample release (i.e., release of enzyme).

Figure 10 is a graph showing ultrasound-mediated release of oligonucleotide from the erythrocyte vehicle. X-axis: power density (W/cm^2), left hand Y-axis: percentage cell lysis; right hand Y-axis: % oligo release. Filled squares represent control cell lysis, filled triangles represent cell lysis and filled diamonds represent % oligo released.

Figure 11 shows flow cytometry profiles obtained following analysis of human erythrocytes loaded with fluorescein-labelled anti-rat IgG using (i) electrosensitisation (pre-sensitisation) -hypoosmotic dialysis - electrosensitisation protocol (ES+HD+ES; ---; "pre-sensitisation"), (ii) hypoosmotic dialysis alone (HD; — ;"dialysis") or (iii) a sonoporation -

hypoosmotic dialysis - electrosensitisation (SP+HD+ES; ····; "sonoporation") protocol. The X-axis represents the fluorescence intensity and the Y-axis represents the number of counts detected at any given fluorescence intensity.

Figure 12 shows ultrasound-mediated release of antibody payload (anti-von Willebrand factor antibody) from loaded and sensitised human cells diluted in normal human cells at 40% hematocrit. Continuous wave ultrasound at 5W/cm^2 is used. X-axis: ultrasound exposure time (minutes); Y-axis: antibody payload released (%). The target cells were circulated through a system in which the temperature was maintained at 37°C and the flow rate during exposure was 14.5ml/min. Gray bars: control; black bars: test.

DETAILED DESCRIPTION OF THE INVENTION

SENSITISATION/PRE-SENSITISATION

In the method of the present invention, cells are subject to at least two sensitisation steps, one of which must be performed prior to, or concomitant with, the loading step, preferably prior to the loading step. For this reason, the first sensitisation step is referred to herein as a pre-sensitisation step. The purpose of the pre-sensitisation step is to enhance the loading of the agent, although an increase in sensitivity to ultrasound mediated lysis may also be achieved. The additional sensitisation steps may be performed at any stage in the process after the pre-sensitisation step. The purpose of the additional sensitisation step or steps is to increase the sensitivity of the cells to ultrasound.

In two particular embodiments that are exemplified herein, a second sensitisation step is carried out either after the pre-sensitisation step but prior to dialysis loading, or after dialysis loading. Further sensitisation steps may be performed as required.

Generally, the sensitisation steps and the loading step are temporally separated. For example, cells are typically allowed to rest in buffer, such as PBS/Mg/glucose buffer, for at least 30 mins, preferably at least 60 mins, after a pre-sensitisation step to allow the cells to

recover prior to loading or further sensitisation steps. It may be desirable to allow cells to rest for several hours, such as overnight, after the loading step.

The pre-sensitisation step increases the efficiency of loading of an agent into a red blood cell, compared to a red blood cell which has not been subject to pre-sensitisation. The pre-sensitisation may take the form of an electrosensitisation step, as described below. Alternatively, or in addition, the pre-sensitisation may be effected by the use of ultrasound, as described below and shown in the Examples. Other methods may be used to pre-sensitise cells and enhance loading efficiency. For example, electromagnetic radiation such as microwaves, radio waves, gamma rays and X-rays may be used. In addition, the use of chemical agents such as DMSO and pyrrolidinone may be envisaged. Furthermore, thermal energy may be imparted on the red blood cells to pre-sensitise them. This may be achieved by raising the temperature of the red blood cells by conventional means, by heat shock, or by the use of microwave irradiation. In general, any method which allows pores to be formed on the surface membrane of a red blood cell is a suitable candidate for use as a pre-sensitisation step.

Preferably, the sensitisation step comprises an electrosensitisation procedure as described next. We have found that the efficiency of sensitisation for given electrical parameters varies depending on the cell density and it may therefore be necessary to perform a titration of cell density and or electrical parameters to establish the optimum concentration. By way of guidance, we have found that cells sensitised at a density of about 6-8x10^8 cells/ml had good sensitivity to ultrasound.

ELECTROSENSITISATION

The present invention encompasses the use of an electric field for sensitising a red blood cell to ultrasound ("electrosensitisation"). Electrosensitisation may also be used as a means of pre-sensitising red blood cells.

The term "electrosensitisation" encompasses the destabilisation of cells without causing fatal damage to the cells. According to this method, a momentary exposure of a

cell to one or more pulses at high electric field strength results in membrane destabilisation. The strength of the electric field is adjusted up or down depending upon the resilience or fragility, respectively, of the cells being loaded and the ionic strength of the medium in which the cells are suspended.

Electrosensitisation typically involves the use of electric fields which do not possess sufficient energy to electroporate the cells. Electroporation, which facilitates the passage of agents into the cell without significant loss of cellular contents or cell viability, is well known in the art, and apart from the energy levels involved is similar to electrosensitisation. Indeed, cells which are electroporated become electrosensitised. However, electrosensitisation may be carried out at energy levels which are insufficient to electroporate the cell and permit the passage of substances through the cell wall and/or cell membrane. In a highly preferred embodiment of the present invention, electrosensitisation of the red blood cells is carried out at these energy levels.

Electroporation has been used in both *in vitro* and *in vivo* procedures to introduce foreign material into living cells. With *in vitro* applications, a sample of live cells is first mixed with the agent of interest and placed between electrodes such as parallel plates. Then, the electrodes apply an electrical field to the cell/implant mixture. Examples of systems that perform *in vitro* electroporation include the Electro Cell Manipulator ECM600 product, and the Electro Square Porator T820, both supplied by the BTX Division of Genetronics, Inc (see US Patent No 5,869,326).

These known electroporation techniques (both *in vitro* and *in vivo*) function by applying a brief high voltage pulse to electrodes positioned around the treatment region. The electric field generated between the electrodes causes the cell membranes to temporarily become porous, whereupon molecules of the agent of interest enter the cells. In known electroporation applications, this electric field comprises a single square wave pulse on the order of 1kV/cm, of about 100 μs duration. Such a pulse may be generated, for example, in known applications of the Electro Square Porator T820.

Electrosensitisation may be performed in a manner substantially identical to the procedure followed for electroporation, with the exception that lower electric field strengths may be used, as set forth below.

In a preferred aspect of the present invention, the electric field has a strength of from about 0.1 kV /cm to about 10 kV/cm under *in vitro* conditions, more preferably from about 1.5 kV/cm to about 4.0 kV/cm under *in vitro* conditions. Most preferably, the electric field strength is about 3.625kV/cm under *in vitro* conditions.

Preferably the electric field has a strength of from about 0.1 kV/cm to about 10 kV/cm under *in vivo* conditions (see WO97/49450). More preferably, the electric field strength is about 3.625kV/cm under *in vitro* conditions.

Preferably the application of the electric field is in the form of multiple pulses such as double pulses of the same strength and capacitance or sequential pulses of varying strength and/or capacitance. A preferred type of sequential pulsing comprises delivering a pulse of less than 1.5 kV/cm and a capacitance of greater than 5 μF, followed by a pulse of greater than 2.5 kV/cm and a capacitance of less than 2 μF, followed by another pulse of less than 1.5 kV/cm and a capacitance of greater than 5 μF. A particular example is 0.75 kV/cm, 10 μF; 3.625 kV/cm, 1 μF and 0.75 kV/cm, 10 μF.

Preferably the electric pulse is delivered as a waveform selected from an exponential wave form, a square wave form and a modulated wave form.

As used herein, the term "electric pulse" includes one or more pulses at variable capacitance and voltage and including exponential and/or square wave and/or modulated wave forms.

Other electroporation procedures and methods employing electroporation devices are widely used in cell culture, and appropriate instrumentation is well known in the art.

In a particularly preferred embodiment, the following electrosensitisation protocol is used. Cells are suspended in PBS to yield concentrations of about 6-8x10^8 cells/ml and 0.8 ml aliquots are dispensed into sterile electroporation cuvettes (0.4 cm electrode gap) and retained on ice for 10 min. Cells are then exposed to an sensitisation strategy involving delivery of two electric pulses (field strength = 3.625 kV/cm at a capacitance of 1 μF) using a BioRad Gene Pulser apparatus. Cells are immediately washed with PBS containing $MgCl_2$ (4mM) (PBS/Mg) and retained at room temperature for at least 30min in the PBS/Mg buffer at a concentration of 7x10^8 cells/ml to facilitate re-sealing. Optionally, cells are subsequently washed and suspended at a concentration of 7x10^8 cells/ml in PBS/Mg containing 10 mM glucose (PBS/Mg/glucose) for at least 1 hour.

PRE-SENSITISATION USING ULTRASOUND

As noted above, ultrasound may be used to pre-sensitise red blood cells. Such use of ultrasound is also referred to herein as "sonoporation". Exposure of red blood cells to ultrasound is believed to result in non-destructive and transient membrane poration (Miller et al, 1998, *Ultrasonics* 36, 947-952).

As used herein, the term "ultrasound" refers to a form of energy which consists of mechanical vibrations the frequencies of which are so high they are above the range of human hearing. The lower frequency limit of the ultrasonic spectrum may generally be taken as about 20 kHz. Most diagnostic applications of ultrasound employ frequencies in the range 1 and 15 MHz (from *Ultrasonics in Clinical Diagnosis*. Edited by PNT Wells, 2nd. Edition, Publ. Churchill Livingstone [Edinburgh, London & NY, 1977].

Ultrasound has been used in both diagnostic and therapeutic applications. When used as a diagnostic tool ("diagnostic ultrasound"), ultrasound is typically used in an energy density range of up to about 100 mW/cm^2 (FDA recommendation), although energy densities of up to 750mW/cm^2 have been used. In physiotherapy, ultrasound is typically used as an energy source in a range up to about 3 to 4 W/cm^2 (WHO recommendation). In other therapeutic applications, higher intensities of ultrasound may be employed, for example, HIFU at 100W/cm^2 up to 1kW/cm^2 (or even higher) for short periods of time.

The term "ultrasound" as used in this specification is intended to encompass diagnostic, therapeutic and focused ultrasound.

Focused ultrasound (FUS) allows thermal energy to be delivered without an invasive probe (see Morocz *et al.*, 1998 *Journal of Magnetic Resonance Imaging* Vol.8, No.1, pp.136-142. Another form of focused ultrasound is high intensity focused ultrasound (HIFU) which is reviewed by Moussatov *et al.* in *Ultrasonics*, 1998 Vol.36, No.8, pp.893-900 and TranHuuHue *et al.* in *Acustica*, 1997, Vol.83, No.6, pp.1103-1106.

Preferably, the red blood cells are pre-sensitised by exposure to ultrasound that has an energy density in the therapeutic range. In a highly preferred embodiment, treatment is at 2.5W/cm^2 for 5 min using a 1MHz ultrasound head. This combination is however not intended to be limiting. Indeed, various combinations of frequency, energy density and exposure time may be used to pre-sensitise the red blood cells so that their loading efficiency is increased.

LOADING

As used herein, the term "loading" refers to introducing into a red blood at least one agent. The agent may be loaded by becoming internalised by, affixed to the surface of, or anchored into the plasma membrane of a red blood cell. Where the agent is affixed or anchored to the plasma membrane, loading may be achieved by cross-linking the agent to any cell surface molecule. Alternatively, the agent may be conjugated to or fused with an antibody specific for a cell surface molecule.

Loading of a red blood cell with more than one agent may be performed such that the agents are loaded individually (in sequence) or together (simultaneously or concurrently). Loading is generally performed in a separate procedure to the "sensitising" procedure. The agents may be first admixed at the time of contact with the red blood cells or prior to that time.

According to the present invention, red blood cells are loaded either after the pre-sensitisation procedure or after one or more sensitisation procedures, preferably after the cells have rested. In this embodiment, the loading may be performed by any desired technique. Accordingly, the present invention encompasses the sensitisation of a pre-sensitised and loaded cell. It also encompasses the loading of a pre-sensitised and subsequently sensitised cell.

The loading may be performed by a procedure selected from the group consisting of electroporation, iontophoresis, sonoporation, microinjection, calcium precipitation, membrane intercalation, microparticle bombardment, lipid-mediated transfection, viral infection, osmosis, osmotic pulsing, osmotic shock, diffusion, endocytosis, phagocytosis, crosslinking to a red blood cell surface component, chemical crosslinking, mechanical perforation/restoration of the plasma membrane by shearing, single-cell injection or a combination thereof.

Sonoporation as a method for loading an agent into a cell is disclosed in, for example, Miller et al (1998), *Ultrasonics* 36, 947-952.

Iontophoresis uses electrical current to activate and to modulate the diffusion of a charged molecule across a biological membrane, such as the skin, in a manner similar to passive diffusion under a concentration gradient, but at a facilitated rate. In general, iontophoresis technology uses an electrical potential or current across a semipermeable barrier. By way of example, delivery of heparin molecules to patients has been shown using iontophoresis, a technique which uses low current (d.c.) to drive charged species into the arterial wall. The iontophoresis technology and references relating thereto is disclosed in WO 97/49450.

In a highly preferred embodiment, the red blood cell is pre-sensitised by electrosensitisation, and loaded using osmotic shock. If more than one agent is employed, the same or a different technique may be used to load the second agent into the red blood cell.

Preferably the red blood cells of the present invention are pre-sensitised, sensitised and loaded *in vitro* or *ex-vivo*.

Preferably loading is carried out by an osmotic shock procedure. The term "osmotic shock" is intended herein to be synonymous with the term "hypotonic dialysis" or "hypoosmotic dialysis".

A preferred osmotic shock/hypotonic dialysis method is described in the Examples and is based on the method described in Eichler *et al.*, 1986, Res. Exp. Med. 186: 407-412. This preferred method is as follows. Washed red blood cells are suspended in 1 ml of PBS (150 mM NaCl, 5 mM K_2HPO_4/KH_2PO_4; pH 7.4) to obtain a hematocrit of approximately 60%. The suspension is placed in dialysis tubing (molecular weight cut-off 12-14,000; Spectra-Por; prepared as outlined below) and swelling of cells obtained by dialysis against 100 ml of 5 mM K_2HPO_4/KH_2PO_4, pH 7.4 for 90 minutes at 4°C. Resealing is achieved by subsequent dialysis for 15 minutes at 37°C against 100ml of PBS containing 10 mM glucose. Cells are then washed in ice cold PBS containing 10 mM glucose using centrifugation.

Other osmotic shock procedures include the method described in U.S. Pat. No. 4,478,824. That method involves incubating a packed red blood cell fraction in a solution containing a compound (such as dimethyl sulphoxide (DMSO) or glycerol) which readily diffuses into and out of cells, rapidly creating a transmembrane osmotic gradient by diluting the suspension of red blood cell in the solution with a near-isotonic aqueous medium. This medium contains an anionic agent to be introduced (such as inosine monophosphate or a phosphorylated inositol, for example inositol hexaphosphate) which may be an allosteric effector of haemoglobin, thereby causing diffusion of water into the cells with consequent swelling thereof and increase in permeability of the outer membranes of the cells. This increase in permeability is maintained for a period of time sufficient only to permit transport of the anionic agent into the cells and diffusion of the readily-diffusing compound out of the cells. This method is of limited effectiveness where the desired agent to be loaded into cells is not anionic, or is anionic or polyanionic but is not present in the

near-isotonic aqueous medium in sufficient concentration to cause the needed increase in cell permeability without cell destruction.

U.S. Patent No. 4,931,276 and WO 91/16080 also disclose methods of loading red blood cells with selected agents using an osmotic shock technique. Therefore, these techniques can be used to enable loading of red blood cells in the present invention.

Effective agents which may advantageously be loaded into red blood cells using the modified method provided in U.S. Patent No. 4,931,276 include peptides, purine analogues, pyrimidine analogues, chemotherapeutic agents and antibiotic agents. These agents frequently present drug delivery problems. Specific compounds include but are not limited to tryptophan, phenylalanine and other water-soluble amino acid compounds. Several derivatives of the unnatural analogues of the nucleic acid bases adenine, guanine, cytosine and thymine are well known as useful therapeutic agents, e.g. 6-mercaptopurine (6MP) and azathioprine, which are commonly used as immunosuppressants and inhibitors of malignant cell growth, and azidothymidine (AZT) and analogues thereof which are useful as anti-viral agents, particularly in the treatment of AIDS. It has been shown that the action of these unnatural base derivatives is dependent on intra-cellular conversion thereof to phosphorylated forms (Chan *et al.*, 1987, *Pharmacotherapy*, 7: 165;14 177; also Mitsuya *et al.*, 1986, *Proc. Natl. Acad. Sci. U.S.A.*, 83: 1911-1915).

An alternative osmotic shock procedure is described in U.S. Patent No. 4,931,276 which is incorporated herein by reference.

Alternatively, loading may be carried out by a microparticle bombardment procedure. Microparticle bombardment entails coating gold particles with the agent to be loaded, dusting the particles onto a 22 calibre bullet, and firing the bullet into a restraining shield made of a bullet-proof material and having a hole smaller than the diameter of the bullet, such that the gold particles continue in motion toward cells *in vitro* and, upon contacting these cells, perforate them and deliver the payload to the cell cytoplasm.

It will be appreciated by one skilled in the art that combinations of methods may be used to facilitate the loading of a red blood cell with agents of interest according to the invention. Likewise, it will be appreciated that a first and second agent, may be loaded concurrently or sequentially, in either order, into a red blood cell in any method of the present invention.

As would be apparent to one of skill in the art, any one or more of the above techniques can be used to load red blood cells for use in the invention, either prior to, simultaneously with, separate from or in sequence to the sensitisation procedure. For example, U.S. Patent No. 4,224,313 discloses a process for preparing a mass of loaded cells suspended in a solution by increasing the permeability of the cell membranes by osmotic pressure or an electric field, or both, loading agents by passage from a solution through the membranes of increased permeability, restoring the original permeability by sealing the membranes by regeneration effect, and separating the cells from the solution in which they were suspended. In that procedure, the agents in solution which are to be loaded include i) a pharmaceutical substance which reacts chemically or physically with substances in the extracellular milieu and which, when loaded into the cell, would prematurely destroy the cell membranes, and ii) at least one blood-compatible sugar and protein capable of providing hydrogen bridge bonding- or of entering into covalent bonds with the pharmaceutical substance, thereby inhibiting the reaction of the pharmaceutical substance with the cell membranes.

It will be appreciated by one skilled in the art that combinations of methods may be used to facilitate the loading of a red blood cell with agents of interest according to the invention. Likewise, it will be appreciated that a first and second agent, may be loaded concurrently or sequentially, in either order, into a red blood cell in any method of the present invention.

The concentration of agent used in the loading procedure may need to be optimised. For example, we have shown that an FITC-IgG antibody achieves good loading at concentrations of 0.1 mg/ml to 2 mg/ml.

Preferably loading takes place over a period of at least 30 mins, more preferably about 90 mins.

SELECTIVE RELEASE USING ULTRASOUND

According to the invention, agents which are loaded into a red blood cell are released from the red blood cell and into their surroundings, in this case at or into the target site, tissue or cell, by the application of ultrasound directed at a target site, tissue and/or cell. Furthermore, the agent may be delivered to the target site by application of ultrasound to vessels, for example, blood vessels, feeding the target site. A general discussion on ultrasound, including different types of ultrasound (for example, diagnostic, therapeutic and focussed ultrasound), is presented above.

Preferably, a combination of diagnostic ultrasound and a therapeutic ultrasound is employed to effect selective release. This combination is not intended to be limiting, however, and the skilled reader will appreciate that any variety of combinations of ultrasound may be used. Additionally, the energy density, frequency of ultrasound, and period of exposure may be varied. What is important is that the application of ultrasound is able to selectively disrupt the sensitised red blood cells to effect release of agent, without substantially disrupting or damaging endogenous red blood cells.

Preferably the ultrasound is applied to a target cell or target tissue with sufficient strength to disrupt loaded and sensitised red blood cells but without damaging the target tissue or surrounding tissues. In this context, the term "damage or damaging" does not include a transient permeabilisation of the target site by the ultrasound energy source. Such a permeabilisation may facilitate uptake of the released payload at the target site.

Preferably the exposure to an ultrasound energy source is at a power density of from about 0.05 to about 100 Wcm^{-2}. Even more preferably, the exposure to an ultrasound energy source is at a power density of from about 1 to about 15 Wcm^{-2}.

Preferably the exposure to an ultrasound energy source is at a frequency of from about 0.015 to about 10.0 MHz. More preferably the exposure to an ultrasound energy source is at a frequency of from about 0.02 to about 5.0 MHz.

Preferably the exposure is for periods of from about 10 milliseconds to about 60 minutes. More preferably the exposure is for periods of from about 1 second to about 5 minutes. Depending on the amount of agent which it is desired to release, however, the exposure may be for a longer duration, for example, for 15 minutes.

Particularly preferably the patient is exposed to an ultrasound energy source at an acoustic power density of from about 0.05 Wcm^{-2} to about 10 Wcm^{-2} with a frequency ranging from about 0.015 to about 10 MHz (see WO 98/52609). However, alternatives are also possible, for example, exposure to an ultrasound energy source at an acoustic power density of above $100Wcm^{-2}$, but for reduced periods of time, for example, $1000Wcm^{-2}$ for periods in the millisecond range or less.

Use of ultrasound is advantageous as, like light, it can be focused accurately on a target. Moreover, ultrasound is advantageous as it can be focussed more deeply into tissues unlike light. It is therefore better suited to whole-tissue penetration (such as but not limited to a lobe of the liver) or whole organ (such as but not limited to the entire liver or an entire muscle, such as the heart) delivery of agents according to the present invention. In addition, ultrasound may induce a transient permeabilisation of the target site so that uptake of a released payload is facilitated at the target site. Another important advantage is that ultrasound is a non-invasive stimulus which is used in a wide variety of diagnostic and therapeutic applications. By way of example, ultrasound is well known in medical imaging techniques and, additionally, in orthopaedic therapy. Furthermore, instruments suitable for the application of ultrasound to a subject vertebrate are widely available and their use is well known in the art.

In methods of the invention, release of the agent is effected by exposure of red blood cells either *in vitro* or *ex-vivo* to an effective amount of a diagnostic ultrasound energy source or a therapeutic ultrasound energy source as described in US Patent No.

5558092 and WO94/28873. The agent, which is released from a red blood cell for use in the present invention may be referred to as the "payload" of that cell.

Preferably the agent is released from the red blood cell by treatment of a target site, tissue or cell with ultrasound.

The selective release of the agent at the target site can be determined by observing a) the amount which has been released at the target site, tissue or cell and b) its effect on the target site, tissue or cell, the latter determining whether its delivery should increase, decrease or be discontinued.

BLOOD CELLS

In one embodiment of the present invention, the red blood cells which may be loaded and administered to a vertebrate according to the invention are ideally obtained from the intended recipient individual prior to the procedure so as to ensure complete immuno-compatibility. Alternatively, cells are obtained from a second individual of the same species as the recipient; in such a case, the second individual must share the blood type of the intended recipient or must have an immuno-neutral blood type, such as type O in humans. Alternatively, the red blood cell may have its immunological determinants masked by a substance such as PEG and/or modified, for example by one or more enzymes.

As used herein, the term "red blood cell" refers to a living, enucleate red blood cell (i.e., a mature erythrocyte) of a vertebrate.

Preferably the red blood cell is a mammalian red blood cell, advantageously a human red blood cell. As used herein, the term "mammal" refers to a member of the class Mammalia including, but not limited to, a rodent, lagomorph, pig or primate. Preferably, the mammal is a human.

As used herein the term "introducing" includes but is not limited to the administration of a red blood cell and/or an agent into a vertebrate.

As used herein in reference to administration of an agent to a vertebrate, the term "introducing" includes but is not limited to causing the agent to enter the circulatory system of the vertebrate by transfusion or to infusing an agent to a target site. It is contemplated that a hollow needle, such as a hypodermic needle or cannula, is inserted through the wall of a blood vessel (e.g., a vein or artery) and the red blood cell is either injected using applied pressure or allowed to diffuse or otherwise migrate into the blood vessel. It is understood that the diameter of the needle is sufficiently large and the pressure sufficiently light to avoid damage of the cell by shear forces. Preferably, introduction of a red blood cell into a vertebrate in a method of the invention is intra-arterial or intravenous. Methods of blood cell transfusion are well known in the art.

As used herein, the term "red blood cell delivery vector" means a red blood cell that has been electrosensitised and loaded with one or more agents according to the methods of the invention and can be used to deliver the agent to a vertebrate. The red blood cell delivery vector is typically made to release the agent at a site of interest in the vertebrate using ultrasound as described above.

AGENT

As used herein, the term "agent" includes but is not limited to an atom or molecule, wherein a molecule may be inorganic or organic, a biological effector molecule and/or a nucleic acid encoding an agent such as a biological effector molecule, a protein, a polypeptide, a peptide, a nucleic acid, a peptide nucleic acid (PNA), a virus, a virus-like particle, a nucleotide, a ribonucleotide, a synthetic analogue of a nucleotide, a synthetic analogue of a ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analogue, a modified amino acid, a modified amino acid analogue, a steroid, a proteoglycan, a lipid, a fatty acid and a carbohydrate. An agent may be in solution or in suspension (e.g., in crystalline, colloidal or other particulate form). The agent may be in the form of a monomer, dimer, oligomer, etc, or otherwise in a complex.

The agent may be an imaging agent, by which term is meant an agent which may be detected, whether *in vitro* in the context of a tissue, organ or organism in which the agent is located. The imaging agent may emit a detectable signal, such as light or other electromagnetic radiation. The imaging agent may be a radio-isotope as known in the art, for example ^{32}P or ^{35}S or ^{99}Tc, or a molecule such as a nucleic acid, polypeptide, or other molecule as explained below conjugated with such a radio-isotope. The imaging agent may be opaque to radiation, such as X-ray radiation. The imaging agent may also comprise a targeting means by which it is directed to a particular cell, tissue, organ or other compartment within the body of an animal. For example, the agent may comprise a radiolabelled antibody specific for defined molecules, tissues or cells in an organism.

The imaging agent may be combined with, conjugated to, mixed with or combined with, any of the agents disclosed herein.

It will be appreciated that it is not necessary for a single agent to be used, and that it is possible to load two or more agents for into the vehicle. Accordingly, the term "agent" also includes mixtures, fusions, combinations and conjugates, of atoms, molecules etc as disclosed herein. For example, an agent may include but is not limited to: a nucleic acid combined with a polypeptide; two or more polypeptides conjugated to each other; a protein conjugated to a biologically active molecule (which may be a small molecule such as a prodrug); or a combination of a biologically active molecule with an imaging agent.

As used herein, the term "biological effector molecule" or "biologically active molecule" refers to an agent that has activity in a biological system, including, but not limited to, a protein, polypeptide or peptide including, but not limited to, a structural protein, an enzyme, a cytokine (such as an interferon and/or an interleukin) an antibiotic, a polyclonal or monoclonal antibody, or an effective part thereof, such as an Fv fragment, which antibody or part thereof may be natural, synthetic or humanised, a peptide hormone, a receptor, a signalling molecule or other protein; a nucleic acid, as defined below, including, but not limited to, an oligonucleotide or modified oligonucleotide, an antisense oligonucleotide or modified antisense oligonucleotide, cDNA, genomic DNA, an artificial or natural chromosome (e.g. a yeast artificial chromosome) or a part thereof, RNA,

including mRNA, tRNA, rRNA or a ribozyme, or a peptide nucleic acid (PNA); a virus or virus-like particles; a nucleotide or ribonucleotide or synthetic analogue thereof, which may be modified or unmodified; an amino acid or analogue thereof, which may be modified or unmodified; a non-peptide (e.g., steroid) hormone; a proteoglycan; a lipid; or a carbohydrate. If the biological effector molecule is a polypeptide, it may be loaded directly into a red blood cell of the invention; alternatively, a nucleic acid molecule bearing a sequence encoding the polypeptide, which sequence is operatively linked to transcriptional and translational regulatory elements active in a cell at the target site, may be loaded. Small molecules, including inorganic and organic chemicals, are also of use in the present invention. In a particularly preferred embodiment of the invention, the biologically active molecule is a pharmaceutically active agent, for example, an isotope.

Particularly useful classes of biological effector molecules include, but are not limited to, antibiotics, anti-inflammatory drugs, angiogenic or vasoactive agents, growth factors and cytotoxic agents (e.g., tumour suppressers). Cytotoxic agents of use in the invention include, but are not limited to, diptheria toxin, Pseudomonas exotoxin, cholera toxin, pertussis toxin, and the prodrugs peptidyl-p-phenylenediamine-mustard, benzoic acid mustard glutamates, ganciclovir, 6-methoxypurine arabinonucleoside (araM), 5-fluorocytosine, glucose, hypoxanthine, methotrexate-alanine, N-[4-(a-D-galactopyranosyl) benyloxycarbonyl]-daunorubicin, amygdalin, azobenzene mustards, glutamyl p-phenylenediamine mustard, phenolmustard-glucuronide, epirubicin-glucuronide, vinca-cephalosporin,phenylenediamine mustard-cephalosporin, nitrogen-mustard-cephalosporin, phenolmustard phosphate, doxorubicin phosphate, mitomycin phosphate, etoposide phosphate, palytoxin-4-hydroxyphenyl-acetamide, doxorubicin-phenoxyacetamide, melphalan-phenoxyacetamide, cyclophosphamide, ifosfamide or analogues thereof . If a prodrug is loaded in inactive form, a second biological effector molecule may be loaded into the red blood cell of the present invention. Such a second biological effector molecule is usefully an activating polypeptide which converts the inactive prodrug to active drug form, and which activating polypeptide is selected from the group that includes, but is not limited to, viral thymidine kinase (encoded by Genbank Accession No. J02224), carboxypeptidase A (encoded by Genbank Accession No. M27717), α-galactosidase (encoded by Genbank Accession No. M13571), ß-glucuronidase (encoded by Genbank

Accession No. M15182), alkaline phosphatase (encoded by Genbank Accession No. J03252 J03512), or cytochrome P-450 (encoded by Genbank Accession No. D00003 N00003), plasmin, carboxypeptidase G2, cytosine deaminase, glucose oxidase, xanthine oxidase, ß-glucosidase, azoreductase, t-gutamyl transferase, ß-lactamase, or penicillin amidase. Preferably, the polypeptide capable of activating a prodrug is DT diaphorase. Either the polypeptide or the gene encoding it may be loaded; if the latter, both the prodrug and the activating polypeptide may be encoded by genes on the same recombinant nucleic acid construct.

Preferably the biological effector molecule is selected from the group consisting of a protein, a polypeptide, a peptide, a nucleic acid, a virus, a virus-like particle, a nucleotide, a ribonucleotide, a synthetic analogue of a nucleotide, a synthetic analogue of a ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analogue, a modified amino acid, a modified amino acid analogue, a steroid, a proteoglycan, a lipid and a carbohydrate or a combination thereof (e.g., chromosomal material comprising both protein and DNA components or a pair or set of effectors, wherein one or more convert another to active form, for example catalytically).

The present invention advantageously employs agents which are not able to diffuse through an intact erythrocyte cell wall by passive or active means. However, the delivery of agents which diffuse at a certain rate through the erythrocyte cell wall is contemplated, particularly where increased delivery of the agent at a particular time or location is desirable. Increased delivery may be achieved by ultrasound administation at the approriate time or location.

The agents, including biological effector molecules, may also be delivered into cells as fusions (for example, protein or polypeptide fusions) or conjugates with a protein capable of crossing the plasma membrane and/or the nuclear membrane. Preferably, the agent/biological effector molecule is fused or conjugated to a domain or sequence from such a protein responsible for the translocational activity. Preferred translocation domains and sequences include domains and sequences from the HIV-1-trans-activating protein (Tat), *Drosophila* Antennapedia homeodomain protein and the herpes simplex-1 virus

VP22 protein. By this means, the agent/biological effector molecule is able to enter the cell or its nucleus when released in the vicinity of the cell using the methods described herein.

Exogenously added HIV-1-trans-activating protein (Tat) can translocate through the plasma membrane and to reach the nucleus to transactivate the viral genome. Translocational activity has been identified in amino acids 37-72 (Fawell et al., 1994, *Proc. Natl. Acad. Sci. U. S. A.* 91, 664-668), 37-62 (Anderson et al., 1993, *Biochem. Biophys. Res. Commun.* 194, 876-884) and 49-58 (having the basic sequence RKKRRQRRR) of HIV-Tat. Vives et al. (1997), *J Biol Chem* 272, 16010-7 identified a sequence consisting of amino acids 48-60 (CGRKKRRQRRRPPQC), which appears to be important for translocation, nuclear localisation and trans-activation of cellular genes. Intraperitoneal injection of a fusion protein consisting of β-galactosidase and a HIV-TAT protein transduction domain results in delivery of the biologically active fusion protein to all tissues in mice (*Schwarze* et al., 1999, Science 285, 1569-72)

The third helix of the *Drosophila* Antennapedia homeodomain protein has also been shown to possess similar properties (reviewed in Prochiantz, A., 1999, *Ann N Y Acad Sci*, 886, 172-9). The domain responsible for translocation in Antennapedia has been localised to a 16 amino acid long peptide rich in basic amino acids having the sequence RQIKIWFQNRRMKWKK (Derossi, et al., 1994, *J Biol Chem*, 269, 10444-50). This peptide has been used to direct biologically active substances to the cytoplasm and nucleus of cells in culture (Theodore, et al., 1995, *J. Neurosci* 15, 7158-7167). Cell internalization of the third helix of the Antennapedia homeodomain appears to be receptor-independent, and it has been suggested that the translocation process involves direct interactions with membrane phospholipids (Derossi et al., 1996, J Biol Chem, 271, 18188-93). The VP22 tegument protein of herpes simplex virus is capable of intercellular transport, in which VP22 protein expressed in a subpopulation of cells spreads to other cells in the population (Elliot and O'Hare, 1997, *Cell* 88, 223-33). Fusion proteins consisting of GFP (Elliott and O'Hare, 1999, *Gene Ther* 6, 149-51), thymidine kinase protein (Dilber et al., 1999, *Gene Ther* 6, 12-21) or p53 (Phelan et al., 1998, *Nat Biotechnol* 16, 440-3) with VP22 have been targeted to cells in this manner.

Particular domains or sequences from proteins capable of translocation through the nuclear and/or plasma membranes may be identified by mutagenesis or deletion studies. Alternatively, synthetic or expressed peptides having candidate sequences may be linked to reporters and translocation assayed. For example, synthetic peptides may be conjugated to fluoroscein and translocation monitored by fluorescence microscopy by methods described in Vives et al. (1997), *J Biol Chem* 272, 16010-7. Alternatively, green fluorescent protein may be used as a reporter (Phelan et al., 1998, *Nat Biotechnol* 16, 440-3).

Any of the domains or sequences or as set out above or identified as having translocational activity may be used to direct the agents (including biological effector molecules) into the cytoplasm or nucleus of a cell.

NUCLEIC ACID

A nucleic acid of use in the invention may comprise a viral or non-viral DNA or RNA vector, where non-viral vectors include, but are not limited to, plasmids, linear nucleic acid molecules, artificial chromosomes, condensed particles and episomal vectors. Expression of heterologous genes has been observed after injection of plasmid DNA into muscle (Wolff J. A. *et al.*, 1990, Science, 247: 1465-1468; Carson D.A. *et al.*, US Patent No. 5,580,859), thyroid (Sykes *et al.*, 1994, Human Gene Ther., 5: 837-844), melanoma (Vile *et al.*, 1993, Cancer Res., 53: 962-967), skin (Hengge *et al.*, 1995, Nature Genet., 10: 161-166), liver (Hickman *et al.*, 1994, Human Gene Therapy, 5: 1477-1483) and after exposure of airway epithelium (Meyer *et al.*, 1995, Gene Therapy, 2: 450-460).

As used herein, the term "nucleic acid" is defined to encompass DNA and RNA or both synthetic and natural origin which DNA or RNA may contain modified or unmodified deoxy- or dideoxy- nucleotides or ribonucleotides or analogues thereof. The nucleic acid may exist as single- or double-stranded DNA or RNA, an RNA/DNA heteroduplex or an RNA/DNA copolymer, wherein the term "copolymer" refers to a single nucleic acid strand that comprises both ribonucleotides and deoxyribonucleotides.

The term "synthetic", as used herein, is defined as that which is produced by *in vitro* chemical or enzymatic synthesis.

Therapeutic nucleic acid sequences useful according to the methods of the invention include those encoding receptors, enzymes, ligands, regulatory factors, and structural proteins. Therapeutic nucleic acid sequences also include sequences encoding nuclear proteins, cytoplasmic proteins, mitochondrial proteins, secreted proteins, plasmalemma-associated proteins, serum proteins, viral antigens, bacterial antigens, protozoal antigens and parasitic antigens. Therapeutic nucleic acid sequences useful according to the invention also include sequences encoding proteins, lipoproteins, glycoproteins, phosphoproteins and nucleic acids (e.g., RNAs such as ribozymes or antisense nucleic acids). Ribozymes of the hammerhead class are the smallest known, and lend themselves both to *in vitro* synthesis and delivery to cells (summarised by Sullivan, 1994, J. Invest. Dermatol., 103: 85S-98S; Usman *et al.*, 1996, Curr. Opin. Struct. Biol., 6: 527-533). Proteins or polypeptides which can be expressed by nucleic acid molecules delivered according to the present invention include hormones, growth factors, neurotransmitters, enzymes, clotting factors, apolipoproteins, receptors, drugs, oncogenes, tumour antigens, tumour suppressers, structural proteins, viral antigens, parasitic antigens and bacterial antigens. The compounds which can be incorporated are only limited by the availability of the nucleic acid sequence encoding a given protein or polypeptide. One skilled in the art will readily recognise that as more proteins and polypeptides become identified, their corresponding genes can be cloned into the gene expression vector(s) of choice, administered to a tissue of a recipient patient or other vertebrate, and expressed in that tissue.

DELIVERY OF AGENTS

The method of the present invention is useful for the delivery of agents to a selected site in a vertebrate body, whether an organ, part of an organ or otherwise, in the presence or absence of specific targeting means. This is achieved, as set out above, by the selective disruption by ultrasound at the selected target site of electrosensitised red blood cells loaded with the agent of choice.

Agents useful for use in the present invention are set out above. Preferred agents include those useful for imaging of tissues *in vivo* or *ex vivo*. For example, imaging agents, such as antibodies which are specific for defined molecules, tissues or cells in an organism, may be used to image specific parts of the body by releasing them at a desired location using ultrasound. This allows imaging agents which are not completely specific for the desired target, and which might otherwise lead to more general imaging throughout the organism, to be used to image defined tissues or structures. For example, an antibody which is capable of imaging endothelial tissue may be used to image liver vasculature by releasing the antibody selectively in the liver by applying ultrasound thereto.

KITS

The invention also encompasses a number of kits. Some of the kits comprise partially or fully treated red blood cells. Other kits provide a red blood cell, an agent and packaging materials therefor together with instructions for carrying out the methods of the invention.

A kit designed for the easy delivery of an agent to a recipient vertebrate, whether in a research of clinical setting, is encompassed by the present invention. A kit takes one of several forms, as follows:

A kit for the delivery of an agent to a subject vertebrate comprises red blood cells and the agent and instructions for performing the method of the present invention. Alternatively, the red blood cells are supplied loaded with the agent for convenience of use by the purchaser. In the latter case, the cells are supplied in sensitised form, ready for rapid use or pre-sensitised and loaded but needing a final sensitisation step.

The cells of the kit are typically species-specific to the vertebrate of interest, such as a primate, including a human, canine, rodent, pig or other, as desired; in other words, the cells are of like species with the intended recipient. The cells of the kit are, additionally, specific to the blood type of the intended recipient organism, as needed. Optionally, the kit

comprises one or more buffers for cell sensitisation, washing, re-suspension, dilution and/or administration to a vertebrate. Appropriate buffers are selected from the group that includes low ionic strength saline, physiological buffers such as PBS or Ringer's solution, cell culture medium and blood plasma or lymphatic fluid. The kit additionally comprises packaging materials (such as tubes, vials, bottles, or sealed bags or pouches) for each individual component and an outer packaging, such as a box, canister or cooler, which contains all of the components of the kit. The kit is shipped refrigerated. Optionally, non-cellular components are supplied at room temperature or frozen, as needed to maintain their activity during storage and shipping. They may be in liquid or dry (i.e., powder) form.

A second kit of the invention comprises an agent such as a biological effector molecule, instructions for performing the method of the present invention and, optionally a sensitising device and buffers therefor (e.g., saline or other physiological salt buffer, culture medium, plasma or lymphatic fluid). In addition, the kit contains appropriate packaging materials, as described above. The individual components may be supplied in liquid or dry (i.e., powder) form, and may be at room temperature, refrigerated or frozen as needed to maintain their activity during storage and shipping. Red blood cells for use with this kit may be obtained independently (for example, they may be harvested from the intended recipient vertebrate).

A preferred aspect of the invention is a kit comprising a red blood cell which is loaded with an agent, and packaging materials therefor.

Preferably, a kit as described above further comprises an apparatus for applying the sensitising procedure.

Preferably a kit of the invention further comprises polyethylene glycol. Preferably the kit further comprises a liquid selected from a buffer, diluent or other excipient. More preferably the liquid is selected from a saline buffer, a physiological buffer and plasma.

- Another aspect of the invention is a physiological composition comprising a red blood cell delivery vector of the invention comprising a biological effector molecule

admixed with a physiologically compatible buffer. As used herein, the term "physiologically compatible buffer" or "physiological buffer" is defined as a liquid composition which, when placed in contact with living cells, permits the cells to remain alive over a period of minutes, hours or days. As such, a physiological buffer is substantially isotonic with the cell, such that cell volume does not change more than 20% due to differences in internal and external ionic strength. Non-limiting examples of physiologically compatible buffers or physiological buffers include dilute saline, which may be buffered (e.g., Hanks' buffered saline or phosphate buffered saline), or other physiological salts (e.g., Ringer's solution), dilute glucose, sucrose or other sugar, dilute glycerol with- or without salts or sugars, cell culture media as are known in the art, serum and plasma.

Preferably, the red blood cell of the physiological composition is a human cell.

EXAMPLES

Example 1: Loading of RBC with Oligonucleotides

In the following example three protocols for the loading and sensitisation of red blood cells (RBC/erythrocytes) are demonstrated and compared.

The first procedure demonstrates loading and sensitisation of red blood cells by exponential wave electric or square wave electric field pulse loading. Such electric pulses used for loading or sensitisation are abbreviated as ES. The second procedure consists of loading and sensitisation of red blood cells by a combination of electrosensitisation followed by hypoosmotic dialysis loading (HD, dialysis or osmotic loading). The combination is abbreviated as ES+HD. The third procedure consists of loading and sensitisation of red blood cells by a method comprising electrosensitisation (pre-sensitisation), followed by hypoosmotic dialysis, overnight rest and further treatment of the cells by electrosensitisation. This combination is abbreviated as ES+HD+ES.

In the first procedure, red blood cells are loaded with an oligonucleotide by a conventional electroporation procedure, as described in the prior art, using exponential wave electric pulses. Briefly, human blood was harvested by venipuncture and washed twice in PBS (phosphate buffered saline) by centrifugation. Cells were suspended in PBS containing 60 μg/ml of a random 30-mer FITC-labelled oligonucleotide to yield concentrations of $3.5x10^8$ cells/ml and 0.8 ml aliquots were dispensed into sterile electroporation cuvettes (0.4 cm electrode gap) and retained on ice for 10 min. Cells were then exposed to an electroporation strategy involving delivery of two electric pulses (field strength = 3.625 kV/cm at a capacitance of 1 μF) using a BioRad Gene Pulser apparatus. Cells were immediately washed with PBS containing $MgCl_2$ (4mM) (PBS/Mg) and retained at room temperature for 30min in the PBS/Mg buffer to facilitate re-sealing. Cells were subsequently washed and suspended at a concentration of between 7 and $14x10^8$ cells/ml in PBS/Mg containing 10 mM glucose (PBS/Mg/glucose) for at least 1 hour.

The second procedure employed is essentially as described in our UK patent application 9917416.1, incorporated by reference. Briefly, 10 ml of peripheral venous blood is collected by venipuncture, into lithium heparin anticoagulant containing tubes, and mixed gently. The whole blood is then poured into a polypropylene tube and centrifuged at 300*g* for 15min at room temperature. The plasma and white blood cells (buffy coat) are removed.

1x phosphate buffered saline (PBS, made from Oxoid tablets code BR14a pH7.3) is added and the cells centrifuged at 700*g* for 5min. The supernatant is removed and the pellet of remaining cells resuspended in ice cold 1xPBS. The spin/wash procedure is then repeated once, and cells are suspended in ice-cold PBS at $6x10^8$ cells/ml.

Cells are then electrosensitised by dispensing 800μl of the RBC into sterile electroporation cuvettes, and placed on ice. To electrosensitise the cells, they are exposed to an electric field at 3.625kV/cm, 1μF (2 pulses), in the absence of payload. The RBCs are then removed, and pooled in polypropylene tubes.

Cells are centrifuged once at 700*g* for 5min at room temperature (RT). The cells may be diluted in PBS/$MgCl_2$ (4mM). Cells are then re-suspended in PBS/$MgCl_2$, and centrifuged at 700*g* for 5min, twice. Finally, cells are re-suspended in PBS/$MgCl_2$, at approximately $7x10^8$c/ml, and rested for 30min at room temperature.

Cells are then loaded with oligonucleotide by hypoosmotic dialysis, according to a protocol adapted from Eichler *et al.*, (1986) *Clin. Pharmacol. Ther.* 40:300-303. The following protocol is followed:

1 BUFFERS:

Stock potassium phosphate buffer:

5mM K_2HPO_4 $3H_2O$ (FW 228.2g) ⇒1.141g/L

5mM KH_2PO_4 (MW136.1g) ⇒0.68g/L

Stored at 4°C

Mix as follows:

For a pH7.4 K_2H/KH_2 phosphate buffer ⇒approx. 6.1:3.9 parts

Mix the 2 stock solutions as and when required

Buffer #1 (isoosmotic PBS):

pH7.4 K_2H/KH_2 phosphate buffer

150mM NaCl ⇒ 8.76g/L

Check and adjust pH (1M NaOH)

Buffer #2 (dialysis buffer):

pH7.4 K_2H/KH_2 phosphate buffer

Check and adjust pH (1M NaOH)

Buffer #3 (resealing buffer)

pH7.4 K_2H/KH_2 phosphate buffer

150mM NaCl ⇒ 8.76g/L

10mM glucose ⇒ 1.8g/L

Check and adjust pH (1M NaOH)

2 SPECTRAPOR DIALYSIS TUBING:

1 The 3.5kDa MW cut off tubing, 0.32ml/cm, is used.

2 Preparation: heat at 80°C/30min in 1mM EDTA/2% sodium bicarbonate (Sigma). Rinse well, inside and outside, with ddH_2O.

3 Wash inside and outside with Buffer #1

4 Store submerged in a small amount of Buffer #1 if not used immediately.

3 RBC PREPARATION:

1 Electrosensitised, rested RBC are washed in PBS twice at 700*g* for 5min.

2 For the final wash, cells are washed in buffer #1

3 The cells are manipulated as a suspension of packed cells following removal of final wash supernatants after centrifugation.

4 CELL VOLUME IN TUBING:

1 Protocol recommends 60% haematocrit (HCT). The suspension of packed cells is approximately 75% HCT and is diluted accordingly.

2 Mix cells with the oligonucleotide and buffer #1, to give required final oligonucleotide concentration and volume.

5 DIALYSIS:

1 The tubing is clipped to ensure that the surface area remains constant for the volume of cells.

2 Dialyse RBC (packed cell volume in buffer #1) against buffer #2 for 90min at 4°C.

3 Place membranes in 100-200ml buffer #2, (ensure that the membrane is immersed) in glass beaker with magnetic flea.

4 Place this beaker within another beaker, which contains ice, on the magnetic stirrer, cover with silver foil.

6 Warm up an aliquot of buffer #3 to 37°C.

7 Remove dialysis buffer, replace with the warm resealing buffer #3.

8 Place beaker with dialysis tubing and buffer #3 into a larger beaker anchored by water at 37°C, cover and leave for 15min.

9 Harvest cells into 12ml polypropylene tubes.

10 Wash x3 in ice cold resealing buffer #3 at 300*g*, 10min 4°C.

11 Wash x1 in PBS/Mg/glucose and spin at 700*g*, 5min 4°C.

12 Count cells and resuspend at $7x10^8$c/ml, in PBS/Mg/glucose.

13 Store at 4°C overnight.

In the present example, dialysis is performed in the presence of 10μg of oligonucleotide per ml of cells. Cells are suspended at $7x10^8$ cells/ml.

In the third procedure, cells are prepared as described for the second procedure, but exposed to an additional electrosensitisation step after loading by dialysis, according to the following protocol.

1 Following overnight storage, wash RBC once in PBS 700*g*, 5min 4°C.

2 Count cells and resuspend at $6x10^8$c/ml, in ice cold PBS.

3 Dispense 800μl of the RBC into sterile electroporation cuvettes (0.4cm gap).

4 Place on ice.

5 To electrosensitise: double pulse at 3.625kV/cm, 1μF.

6 Harvest the RBC, pool in a polypropylene tube.

7 Centrifuge once at 700*g* for 5min room temperature (RT). The cells may be diluted in PBS/$MgCl_2$(4mM).

8 Resuspend in PBS/$MgCl_2$, centrifuge at 700*g* for 5min.

9 Repeat step 6

10 Resuspend in PBS/$MgCl_2$, at approximately $7x10^8$c/ml.

11 Rest the cells for 30min at RT.

12 Centrifuge once at 700*g* for 5min room temperature (RT). The cells may be diluted in PBS/$MgCl_2$/glucose.

13 Resuspend the cells in PBS/$MgCl_2$/glucose, centrifuge at 700*g* for 5min.

14 Repeat step 13.

15 Resuspend cells in PBS/$MgCl_2$/glucose at $7x10^8$c/ml.

16 Rest the cells in PBS/$MgCl_2$/glucose for 60min.

Cells prepared according to all three procedures were analysed to determine cell loading levels and subjected to ultrasound disruption. The results are shown in Figure 1.

For the electroporated cells, shown in Figure 1A, the oligonucleotide (oligo) did not bind non-specifically to RBC as the mean fluorescence intensity (MFI) was 1. The MFI is defined as the ratio of fluorescence associated with loaded cells divided by the fluorescence associated with non-specific binding. The electroloaded cells have an increase in fluorescence, with an MFI of 5.6.

Ultrasound sensitivity was measured at 0.75W/cm^2, 3MHz, 30 sec. 0% of control cells lysed, compared with 20% of the electroporated cells.

For cells loaded by electrosensitisation followed by hypoosmotic dialysis (ES+HD), non-specific binding is negligible (MFI=1). Loading by ES+HD results in a marked increase in fluorescence with an MFI of 80. Incorporation of the second electrosensitisation step (ES+HD+ES) has little effect on the level of fluorescence with an MFI of 93 indicating retention of the payload during the procedure.

Ultrasound sensitivity is measured at 3W/cm^2, 1MHz, 35 sec in a TMM (tissue mimicking medium). Cells subjected to only a single electrosensitisation and dialysis procedure (ES + HD) show 28% lysis, whilst cells subjected to the additional electrosensitisation step (ES + HD + ES) show 89% lysis.

Example 2: Loading of RBC with Antibodies

For comparative purposes, antibodies are loaded into RBC by electroporation. The antibody used is a FITC-conjugated anti-vWF antibody (Sigma). The results are shown in Figure 2.

In a first procedure (Figure 2A), cells are loaded as described in Example 1 by exposing 3.5 x10^8 cells/ml to 3 pulses of an exponential wave electric field in the presence

of antibody at 0.25mg/ml. An MFI of 3.98 is observed, with 100% ultrasound sensitivity. However, in this procedure the cell recovery is low, approximately 11%.

In a second procedure (Figure 2B), 10 pulses of a square wave electric field are used, in the presence of 0.5mg/ml antibody (the conditions are optimised for each protocol). Two peaks are seen in the cell population after loading, with an MFI of 1.73 and 184.8. 40% of the cells are recovered, of which 97% are ultrasound sensitive when exposed to ultrasound at an energy of 1.25W/cm^2 using a 3MHz probe for 30s. 0.005pg antibody per cell was recovered

Figure 3 shows the results of hypoosmotic dialysis loading of antibody according to the procedure of Eichler *et al.* and of the present invention (see Example 1). The relative MFI is 15.2 in the absence of electrosensitisation (HD loading alone) compared to an MFI of 61.7 in the presence of electrosensitisation (ES+HD). This demonstrates a dramatic increase in loading.

In both dialysis protocols, 80 to 90% of the cells are recovered. Ultrasound sensitivity is about 30% in the absence of a second sensitisation step (ES + HD). Following the second sensitisation step (ES + HD + ES) there is no apparent leakage/loss of the payload and about 90-100% of the cells are ultrasound sensitive (see table in Example 6). 0.22pg antibody/cell was recovered.

Example 3: Resealing Buffers

An alternative resealing buffer is tested in order to assess any impact on the performance of the method according to the invention. The buffer of Bax *et al.*, (1999) Clinical Science 96:171-178, is compared to the buffer adapted from Eichler *et al.* as used in Example 1 (ES + HD).

As can be seen from Figure 4, the performance of the two buffers is almost identical. In both cases, cells were loaded with FITC conjugated anti-vWF antibody as

described in Example 2, and subjected to a second electrosensitisation procedure in accordance with the present invention.

The mean fluorescence intensities observed for the Eichler and Bax buffers are 186 and 126 respectively. Observed cell losses are 27% and 16%.

Ultrasound sensitivities are measured in a TMM, at $3W/cm^2$, for 35 seconds; cell lysis of 68% and 72% is observed, respectively.

Example 4: Ultrasound-mediated release of antibody payload in a perfused rat kidney system

The release of FITC-conjugated antibody by ultrasound in PBS-perfused kidney tissue is shown in Figure 5. RBC are loaded with FITC-anti-vWF antibody as described in Example 2 above, in accordance with the present invention, and administered to PBS-perfused kidneys, according to the following protocol:

1. Perfuse the rat through the heart with PBS/EDTA until the kidney is clear of blood
2. Remove the dorsal aorta from the heart and insert a gavage needle into the vessel. Tie the needle to the dorsal aorta using suture.
3. Close the dorsal aorta and posterior vena cava just after the junction leading to the kidney.
4. Close the left adrenal artery and vein and both anterior mesenteric and coeliac arteries
5. Close the ureter and the left iliolumbar artery and vein.
6. Create an exit point by inserting a gavage needle into the vena cava just before the liver. Tie the needle using suture.
7. Flush with 10ml PBS/4mM Mg/10mM glucose and check for any leakage.
8. Block the exit point by inserting 2ml syringe into the gavage needle.
9. Load 1ml of $7x10^8$ cells /ml through the dorsal aorta into the kidney.
10. Treat with U/S using 1MHz probe.
11. Incubate the treated kidney for one hour
12. Remove the 2ml syringe and flush through with 2ml PBS/Mg/glucose

13. Collect the flush through for cell counting and ELISA
14. Flush with 50ml of PBS/EDTA
15. Flush with 20ml of 4% neutral buffered formalin (NBF)
16. Remove the U/S-treated kidney and cut it into two half's and fix in NBF
17. Prepare tissue sections (12μm)and stain using Vectastain ABC kit (Vecta Labs) as outlined in the manufacturer's instructions.

Preparation of RBC: dialysed and electrosensitised (ES+HD+ES), antibody loaded erythrocytes:

Rat 1 No ultrasound treatment

Rat 2 Ultrasound treatment at 3W/cm^2 for 40 seconds.

Kidney endothelial cells in glomeruli are labelled by the FITC conjugated anti-vWF antibody after ultrasound treatment to release the antibody, as shown in Figure 5A. In the absence of ultrasound treatment, no staining is observed (Figure 5B).

Example 5: Stability of Loaded Cells

RBC are loaded by dialysis according to the present invention (ES + HD + ES), as described in Examples 1 and 2, with FITC-conjugated antibody. Following the second sensitisation, cells are stored at 7x10^8 cells/ml in SAGM buffer (Blood transfusion service buffer, obtainable from Baxter Health Care). Cells are stored with maximal exclusion of air at 4°C. Maintenance of ultrasound sensitivity, cell numbers and payload are assessed over a period of 35 days.

Figure 6 shows the levels of cell numbers and ultrasound sensitivity in cells on storage. Ultrasound sensitivity, measured at 3W/cm^2, 35 sec, in a TMM, is maintained at or above the starting level of 90% for 25 days, and falls to about 65% after 35 days. Cell numbers are stable over a 30 day period.

Figure 7 shows the retention of payload over 30 days under identical conditions to the above. No loss of payload is observed.

Example 6: Comparison of different sequences of sensitisation and osmotic loading steps

The hypoosmotic dialysis loading protocol described in Example 1 is performed in two different configurations to determine the effect on loading efficiency and susceptibility to ultrasound mediated lysis when the loading step is performed before the second sensitisation step, as in Example 1, and vice versa.

Electrosensitisation steps and dialysis were carried out as described in Example 1, except that the electrosensitisation steps are carried out twice. Ultrasound sensitivity is determined in a TMM as described in Example 1.

Table 1

Sample	% U/S mediated lysis day of load	% U/S mediated lysis following rest overnight	Mean fluorescence intensity
RBC	0	5	-
ES + dialysis (ES + HD)	0	0	66
ES+ dialysis+ ES (ES+HD+ES)	0	90-100%	61.7
ES + ES + dialysis (ES + ES + HD)	84	95	79

The results shown above in Table 1, similar to those obtained in Example 1, indicate that the sequence of the two sensitisations is not critical to obtaining improved sensitivity.

Example 7: Release of payload from loaded and sensitised vehicle in a tissue mimicking system (TMM)

It has been demonstrated that enhanced loading of cells may be achieved by exposing to electric fields in combination with hypoosmotic dialysis loading modalities. In the studies presented in the examples described here, cells were loaded with antibody, enzyme and oligonucleotide by firstly exposing the cells to pre-sensitising electric pulses and subsequently carrying out hypoosmotic loading. The cells were then electrosensitised by exposing to electric pulses. This protocol is defined by ES+HD+ES in the previous examples (see Example 1). In the experiments described here the objective was to demonstrate enhanced loading to confirm earlier examples and to demonstrate ultrasound mediated release of the relevant payload using a tissue mimicking system. In these experiments the target was placed at a distance of 1.3cm from the emitting surface of the ultrasound head and the intervening space was filled with a tissue mimicking material (TMM) which attenuates ultrasound in the same manner as a soft tissue. The TMM chosen for this work has been described by Madsen *et al.* (1998, *Ultrasound Med. & Biol.*, 24, 535-542) and following preparation, care was taken to ensure that the material had a density of 1.03g/ml.

I. Ultrasound-mediated release of antibody from the vehicle

Antibody was loaded into the erythrocytes and sensitisation were carried out using the procedure denoted by ES+HD+ES as described for Example 1. Antibody-loaded sensitised cells were then exposed to ultrasound at a distance of 1.3cm from the emitting surface of the ultrasound head. The intervening space was filled with the TMM as described above and 0.1ml aliquots of $7x10^8$ cells/ml were exposed to ultrasound. In these studies a sheep anti-human von Willebrand factor antibody was employed as the payload in these studies. The amount of antibody in cells and released following treatment with ultrasound was quantified using an ELISA system.

I. Results

In the loading and sensitisation protocol, cells were loaded at a concentration of 1.1mg of antibody per ml of packed cell volume (PCV). 0.1ml aliquots of cells at $7x10^8$ cells/ml were exposed to ultrasound at intensities shown in Figure 7 and samples were analysed for cell lysis by direct counting. In addition, the amount of antibody released following treatment with ultrasound was determined by ELISA analysis of cell supernatants harvested following centrifugation. The results obtained are shown in Figure 7 and they demonstrate that cells were preferentially lysed at ultrasound power densities greater than $2W/cm^2$. Control cells exhibited little or no effect when treated with ultrasound at these power densities. In addition, at and above $2W/cm^2$ antibody payload was detected in supernatants harvested following ultrasound treatment. In addition, when the total amount of antibody released from the cells using ultrasound was compared with that released following hypotonic lysis in 0.01% (v/v) Triton X100 it was found that 77% of the total antibody was released in the former. The remainder could be found in debris that was recovered by centrifugation following ultrasound treatment.

The results demonstrated that treatment of cells with the ES+HD+ES protocol results in sensitivity of that loaded population to ultrasound. Ultrasound-mediated payload release could be achieved using low intensity ultrasound and using conditions which had little or no effect on normal erythrocytes. The results also demonstrate ultrasound-mediated release of payload at a depth of 1.3cm and thereby demonstrating one of the major advantages associated with the use of ultrasound as the releasing stimulus of penetration to depth in tissues. Since all of the antibody incorporated into the ultrasound treatment experiments could be recovered as shown using an ELISA based on payload functionality, this suggested that the ultrasound had no detrimental effect on the functionality

II. Ultrasound-mediated release of enzyme (β-galactosidase) from the vehicle

Cells were harvested, pre-sensitised by exposure to electric pulses and loaded with β-galactosidase (from *Escherichia coli*, Sigma) as described above for antibody loading. Cells were subsequently exposed to sensitising electric pulsing and exposed to ultrasound

at a concentration of $7x10^8$ cells/ml in the TMM system as described above for the antibody-loaded vehicle. Lysates obtained following exposure of the loaded and sensitised vehicle to ultrasound were assayed for β-galactosidase activity at 37^0C using the colorimetric substrate p-nitrophenyl-β-D-galactoside (5mM in 50mM phosphate buffer, pH 7.0). The concentration of p-nitrophenol was determined spectrophotometrically at 450nm and activity was expressed as μmoles of p-nitrophenol produced per minute per ml of sample. Release of enzyme in samples harvested following treatment with ultrasound was expressed as a percentage relative to the amount of enzyme contained in the cells prior to treatment. The latter was determined by measuring the amount of enzyme released from the cells following lysis by freeze-thaw in 5mM phosphate buffer, pH 7.2.

II. Results

In these experiments loaded cells contained approximately 1mg of enzyme per ml of packed cell volume. The results obtained following treatment of these preparations with ultrasound are shown in Figure 8. Samples were treated at the indicated power densities as shown and samples were analysed for cell lysis by cell counting. Lysis increased with increasing power density up to a maximum at about $3W/cm^2$. Exposure of control normal cells to similar ultrasound conditions had little or no effect on cell lysis and this was confirmed by the absence of haemoglobin in supernatants following removal of cells by centrifugation. When supernatants were harvested by centrifugation, following exposure of the sensitised and loaded cells to ultrasound and analysed for enzyme content, it was found that increasing amounts of enzyme were released with increasing power density up to a maximum at $3W/cm^2$.

The results demonstrated cells loaded using the ES+HD+ES protocol are sensitive to ultrasound and the enzyme payload may be released from the vehicle following exposure to low intensity ultrasound. Ultrasound-mediated release of the payload is achieved at 1.3cm from the emitting ultrasound head, indicating that the use of ultrasound for this purpose offers the advantage of penetration to depth in tissues. In addition, since 100% recovery of the enzyme released was achieved (between 2.5-$3W/cm^2$), the ultrasound

stimulus resulting in release of enzyme had no detrimental effect on the functionality of the released payload.

III. Ultrasound-mediated release of oligonucleotide from vehicle

Cells were harvested and pre-sensitised by exposure to electric pulses as described above. Cells were then loaded using the hypoosmotic dialysis procedure described above for antibody loading and a 300μg quantity of oligonucleotide (tamara labelled random 30-mer supplied by Oswel, UK) was mixed with 250μl of packed cells. Samples were then subjected to electrosensitisation electric pulses and subsequently suspended in PBS/$MgCl_2$/glucose at a concentration of $7x10^8$ cells/ml. Samples were exposed to ultrasound using the TMM system described above for antibody and enzyme release and the amount of oligonucleotide released was determined using a spectrofluorimeter (Shimadzu) with excitation set at 540nm and emission set at 590nm. A standard curve was constructed for quantitative determinations and extraction efficiencies were taken into account.

III. Results

In these experiments the maximum amount of oligonucleotide loaded was approximately 300μg of oligonucleotide per ml of packed cell volume.

The results obtained following treatment of these loaded preparations with ultrasound are shown in Figure 9. As with the above two examples, cell lysis of the sensitised and loaded preparation occurs between 2 and 3 W/cm^2. Under these ultrasound conditions there is little or no effect on control erythroyctes. In addition oligonucleotide begins to appear in harvested supernatants between 2 and 3 W/cm^2 demonstrating ultrasound-mediated release of oligonucleotide payload from the vehicle.

Example 8: Enhanced loading of erythrocytes by replacing initial ES step with a sonoporative treatment

The results above demonstrate that cells loaded using the ES+HD+ES protocol are more efficiently loaded than using ES or HD alone. These results also demonstrate release of the payload from the vehicle.

In many of the previous examples sensitisation and loading was achieved by pre-sensitising the cell using electrosensitisation and subsequently processing through hypoosmotic loading protocols and a further exposure to electric pulses (ES+HD+ES). The resulting preparations were efficiently loaded with the relevant payload and the preparations also exhibited sensitivity to ultrasound. Since it was felt that the initial pre-sensitising event, which is reported to create transient poration of the membrane, contributed positively to loading by hypoosmotic loading it was of interest to determine whether or not other porative methods might contribute in a similar manner. Sonoporation represents an alternative technique known to create transient membrane poration. This involves the use of ultrasound and has been reported to aid in creating non-destructive and transient poration of biological membranes (Miller et al, 1998, *Ultrasonics* 36, 947-952). It was therefore of interest to determine whether or not exposure of erythroyctes to ultrasound prior to hypoosmotic loading would contribute positively to loading of those cells and if so, could those cells be rendered sensitive to ultrasound by subsequently exposing them to sensitising electric pulses.

To the above ends, human erythroyctes were harvested and loaded with fluorescein-labelled anti-rat IgG using either the original electrosensitisation (pre-sensitisation) - hypoosmotic dialysis - electrosensitisation protocol (ES+HD+ES), hypoosmotic dialysis alone (HD) and a sonoporation - hypoosmotic dialysis - electrosensitisation (SP+HD+ES) protocol. The former two were performed as described above and the latter consisted of the original ES-HD-ES protocol except that the first ES step was replaced by a sonoporative step.

This involved suspending washed erythrocytes in PBS at a concentration of $7x10^8$ cells/ml. 1.5ml aliquots were then dispensed into individual wells to a 24-well tissue culture plate. Cells were subjected to ultrasound treatment at $2.5W/cm^2$ for 5 min. using a 1MHz ultrasound head. After treatment cells were washed by centrifugation and suspended in $PBS/MgCl_2$. Cells were then processes as for the ES+HD+ES protocol and loading of antibody was assessed using flow cytometry. In addition, the sensitivity of those cells was determined by exposing the cells to ultrasound at $3W/cm^2$ using the TMM system.

Results 8

When cells were treated with the ES+HD+ES protocol and subsequently exposed to ultrasound at $3W/cm^2$ using the TMM over 90% lysis was obtained and this is in agreement with previous results. When cells were treated with the SP+HD+ES protocol and exposed to ultrasound in the above manner 30% lysis occurred. The results demonstrate that the cells have been sensitised using the alternative protocol although not to the same degree as that achieved with the original protocol involving the use of the preliminary pre-sensitising electrosensitisation step.

When loading of the cells was examined using flow cytometry it was found that the loading with HD alone resulted in two peaks of fluorescent cells as shown in Figure 11. This indicated loading of the cells was inefficient since over half of the population of cells remained unloaded or minimally loaded. When cells were loaded using the ES+HD+ES protocol, a major peak shifted to the right was detected and this indicated that almost all (88%) of the cells in the population were maximally loaded (Figure 11). When the protocol employing a sonoporative pre-sensitisation step prior to hypotonic dialysis was analysed on flow cytometry it was found that again, most of the cells resided in a peak shifted well to the right in Figure 11. This again indicated that almost all of the cells (93%) were loaded with fluorescent antibody. However since the shift to the right in this peak was not as great as that found in the sample treated with the ES+HD+ES protocol, the amount of fluorescent antibody associated with the cells in this peak is not as great and this is indicated by the mean fluorescent intensities listed in the Table 2 below. It should also be noted that yields using the sonoporative method were not as high as those from the

alternative two methods. The ultrasound-mediated protocol provides advantage over hypotonic dialysis in terms of loading the full population of cells. Although the sonoporative protocol is not as efficient in terms of payload incorporated it does provide an alternative means of enhancing loading achieved with electrosensitisation (pre-sensitisation) and hypoosmotic dialysis alone.

When cells were loaded with the SP+HD protocol they did not exhibit sensitivity to ultrasound. However, when subsequent exposure to electric pulses was carried out i.e. SP+HD+ES, 30% lysis of the population ($7x10^8$ cells/ml) was observed when treated using the TMM system described above.

Table 2

Treatment	% of cells in Second Peak	MFI of Second Peak
HD (95)	40	32
ES-HD-ES (88)	88	39
SP-HD-ES (60)	90	27

MFI = mean fluorescence intensity which is = fluorescent intensity of sample/fluorescent intensity exhibited as a result of non-specific binding

Values in parentheses represent the yields of cells obtained from each protocol

Example 9: Ultrasound-mediated release of payload from the loaded, sensitised vehicle in a circulating system at 37°C and at high hematocrit (HCT.)

In the above studies it is shown that human erythroyctes can be loaded at high efficiency, sensitised to low intensity ultrasound and ultrasound-mediated disruption and/or payload release can be achieved *in vitro* and in an *ex vivo* perfused rat kidney system. In these systems disruption and/or payload release is demonstrated at $7x10^8$ cells/ml which is approximately equivalent to a 5% HCT. In addition, those studies are performed at room temperature. It is of interest to demonstrate that sensitivity in terms of payload release can be retained at 37°C and at higher HCT. It is also of interest to determine whether or not this

occurs while the target cells are moving through a circulation system in much the same as those circulating *in vivo*.

To these ends human erythrocytes are harvested and loaded with anti-von Willebrand factor antibody as described for Example 7. Following sensitisation cells re-mixed together with normal washed human cells in the proportions of one part $7x10^8$ cells/ml and four parts $4x10^9$ cells/ml. The mixture is introduced into a circulating system consisting of a cylindrical reservoir filled with PBS and maintained at 37°C by circulation. The bottom of the cylinder consists of a light polyethylene sheet through which ultrasound is delivered. The blood is circulated through C-flex tubing (internal diameter 4mm) which passes through the thermostated buffer and the target area of the C-flex tubing is positioned at a distance of 1.3cm from the ultrasound-emitting head. Blood is circulated through the system at a rate of 14.5ml/min. During exposure to ultrasound ($5W/cm^2$ at 1MHz for indicated times), samples are harvested from the system and supernatants are harvested by centrifugation. These are then assayed for antibody using an ELISA assay as described above. The control in these experiments consists of loaded and sensitised cells circulated through the system in the absence of ultrasound. It is also important to note that circulation of normal cells through the system while ultrasound is being delivered results in no apparent damage as determined by the lack of hemoglobin in supernatants following treatment.

Results 9

The results are shown in Figure 12 and they demonstrate that detectable quantities of antibody are released from the vehicle between 2 and 5 minutes treatment with ultrasound. Little or no antibody can be detected in control samples which consist of the loaded and sensitised cells circulated through the system in the absence of ultrasound. The results demonstrate that the ultrasound-sensitisation phenomenon is intact at 37°C and ultrasound-mediated release of payload is achieved at high hematocrit (HCT., i.e., 40%) and in a mobile target system.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method of producing a red blood cell suitable for delivery of an agent to a vertebrate, the method comprising:

(a) providing a red blood cell;

(b) pre-sensitising the red blood cell; and

(c) loading the red blood cell with an agent.

2. A method according to Claim 1, in which the amount of agent that is loaded into a pre-sensitised red blood cell is higher than the amount loaded into a red blood cell which is not pre-sensitised.

3. A method according to Claim 1 or 2, further comprising the step of electrosensitising the cell to render it more susceptible to disruption by exposure to a stimulus, the loading step and the electrosensitisation step being performed in any order.

4 A method for selectively releasing an agent from a red blood cell comprising the steps of:

(a) pre-sensitising a red blood cell;

(b) loading the cell with an agent;

(c) electrosensitising the cell; and

(d) causing the agent to be released from the sensitised cell by applying ultrasound at a frequency and energy sufficient to cause disruption of the sensitised cell but insufficient to cause disruption of unsensitised red blood cells,

in which steps (b) and (c) can be performed in any order.

5. A method for delivering an agent to a target site in a vertebrate, the method comprising a method according to Claim 4, with the further step of introducing the cell into a vertebrate between steps (c) and (d).

6. A method according to Claim 5, in which the red blood cell is PEGylated prior to being introduced into the vertebrate.

7. A method according to Claim 1, 5 or 6, in which the vertebrate is a mammal.

8. A method according to any preceding claim, in which the pre-sensitising step and the electrosensitising step are an *in vitro* or *ex-vivo* procedure.

9. A method according to any preceding claim, in which the pre-sensitising comprises a step of applying an electric field to the red blood cell.

10. A method according to any of Claims 1 to 8, in which the pre-sensitising comprises a step of applying ultrasound to the red blood cell.

11. A method according to any one of the preceding claims, in which the red blood cell is loaded with the agent by hypotonic dialysis.

12. A method according to any of Claims 3 to 11, in which the electrosensitising comprises the step of applying an electric field to the red blood cell.

13. A method according to Claim 12, in which the electric field is from about 0.1 kV/cm to about 10 kV/cm under *in vitro* conditions.

14. A method according to Claim 12 or 13, in which the electric field is applied for between 1 μs and 100 ms.

15. A method according to any one of Claims 3 to 14, in which the electrosensitisation of the red blood cell is performed after the loading of the agent.

16. A method according to any one of Claims 3 to 14, in which the electrosensitisation of the red blood cell is performed before the loading of the agent.

17. A method according to any preceding claim, in which the ultrasound is selected from the group consisting of diagnostic ultrasound, therapeutic ultrasound and a combination of diagnostic and therapeutic ultrasound.

18. A method according to Claim 17, in which the applied ultrasound energy source is at a power level of from about 0.05 W/cm^2 to about 100 W/cm^2.

19. A red blood cell delivery vector which has been pre-sensitised such that it is capable of being loaded with a larger amount of agent than a red blood cell which has not been pre-sensitised.

20. A red blood cell delivery vector according to Claim 19, which has been pre-sensitised by exposure to an electric field and/or ultrasound.

21. A red blood cell delivery vector according to Claim 19 or 20, which is sensitised to render it more susceptible to disruption by exposure to a stimulus.

22. A red blood cell delivery vector according to Claim 19, 20 or 21, which is loaded with an agent to be delivered.

23. A method according to any of Claims 1 to 18, or a red blood cell delivery vector according to Claim 19, 20 or 21, in which the agent is selected from a group consisting of a protein, a polypeptide, a peptide, a nucleic acid, a peptide nucleic acid (PNA), a virus, a virus-like particle, a nucleotide, a ribonucleotide, a deoxyribonucleotide, a modified deoxyribonucleotide, a heteroduplex, a nanoparticle, a synthetic analogue of a nucleotide, a synthetic analogue of a ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analogue, a modified amino acid, a modified amino acid analogue, a steroid, a proteoglycan, a lipid, a fatty acid, an oligosaccharide, a glycoprotein, a carbohydrate, and mixtures, fusions, combinations or conjugates of the above.

24. A method according to any of Claims 1 to 18 and 23, or a red blood cell delivery vector according to Claim 19, 20, 21 or 23, in which the agent is conjugated to, fused to, mixed with or combined with an imaging agent.

25. A red blood cell delivery vector obtainable by a method comprising:

(a) presensitising a red blood cell by electrosensitising the cell;

(b) loading the cell with an agent; and

(c) electrosensitising the cell,

in which steps (b) and (c) can be performed in any order.

26. Use of an electric field and/or ultrasound to increase the efficiency of loading of an agent into a red blood cell.

27. A method of pre-sensitising a red blood cell with an electric field and/or ultrasound such that the amount of agent that is capable of being loaded into the pre-sensitised red blood cell is higher than that which is capable of being loaded into a red blood cell which is not pre-sensitised.

28. A kit comprising a red blood cell made by a method according to any of Claims 1 to 18, 23 and 24 or a red blood cell delivery vector according to any of Claims 19 to 25, packaging materials therefor and instructions for use.

29. A kit comprising a red blood cell, an agent, packaging materials therefor and instructions for use in a method comprising the steps of:

(a) presensitising a red blood cell;

(b) loading the cell with an agent;

(c) electrosensitising the cell; and

(d) causing the agent to be released from the sensitised cell by applying ultrasound at a frequency and energy sufficient to cause disruption of the sensitised cell but insufficient to cause disruption of unsensitised red blood cells,

in which steps (b) and (c) can be performed in any order.

30. A kit comprising a pre-sensitised red blood cell which is loaded with an agent, packaging materials therefor and instructions for use in a method comprising the steps of:

(a) electrosensitising the cell; and

(b) causing the agent to be released from the sensitised cell by applying ultrasound at a frequency and energy to cause disruption of the sensitised cell but insufficient to cause disruption of unsensitised red blood cells.

31. A kit comprising a red blood cell made by a method according to any of Claims 3 to 18, 23 and 24 or a red blood cell delivery vector according to any of Claims 22 to 25, packaging materials therefor and instructions for use comprising the step of causing the agent to be released from the red blood cell delivery vector by applying ultrasound at a

frequency and energy to cause disruption of the red blood cell delivery vector but insufficient to cause disruption of unsensitised red blood cells.

32. A kit according to any of Claims 28 to 31, in which the kit further comprises polyethylene glycol.

33. A kit according to any of Claims 28 to 32, in which the kit further comprises a liquid selected from the group consisting of a buffer, diluent or other excipient.

34. A kit according to Claim 33, in which the liquid is selected from the group consisting of a saline buffer, a physiological buffer, serum and plasma.

35. A pharmaceutical composition comprising a red blood cell made by a method according to any of Claims 1 to 18, 23 and 24 or a red blood cell delivery vector according to any of Claims 19 to 25, together with a pharmaceutically acceptable carrier or diluent.

36. A method and/or a red blood cell delivery vector and/or a kit and/or a pharmaceutical composition substantially as described herein and with reference to the examples and figures.

37. A device for producing a red blood cell delivery vector of the present invention which device comprises:

(a) one or more flow cells and electrosensitisation means;

(b) one or more dialysis systems;

in which the flow cell is linked to the dialysis system by connecting means capable of allowing the transfer of red blood cells from the flow cell to the dialysis system and vice versa.

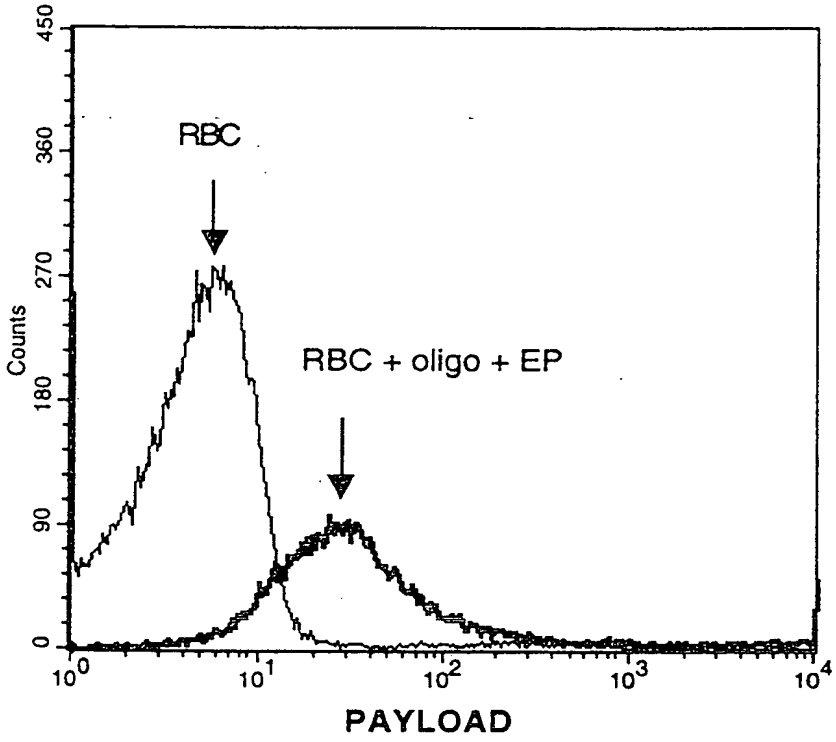
ABSTRACT

LOADING METHOD

We describe a method of producing a red blood cell suitable for delivery of an agent to a vertebrate, the method comprising: (a) providing a red blood cell; (b) pre-sensitising the red blood cell; and (c) loading the red blood cell with an agent. Use of an electric field and/or ultrasound to increase the efficiency of loading of an agent into a red blood cell is also described.

Figure 1

A. ELECTROLOADING OF OLIGONUCLEOTIDE



B. GENDEL DIALYSIS LOADING OF OLIGONUCLEOTIDE

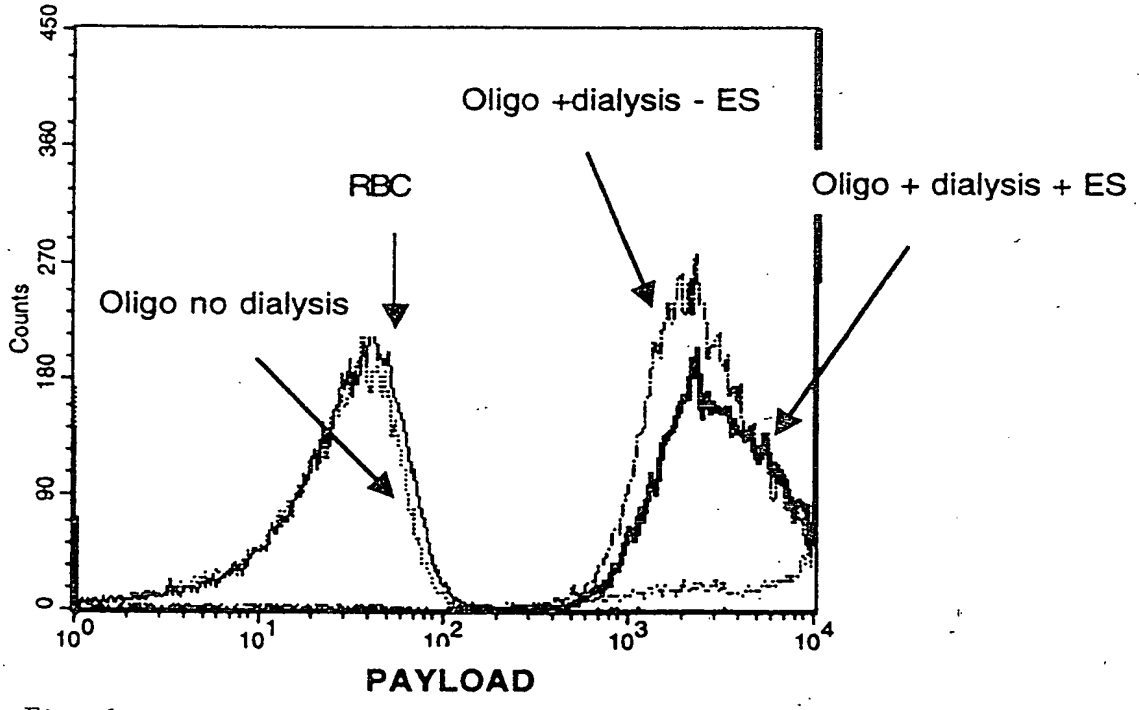
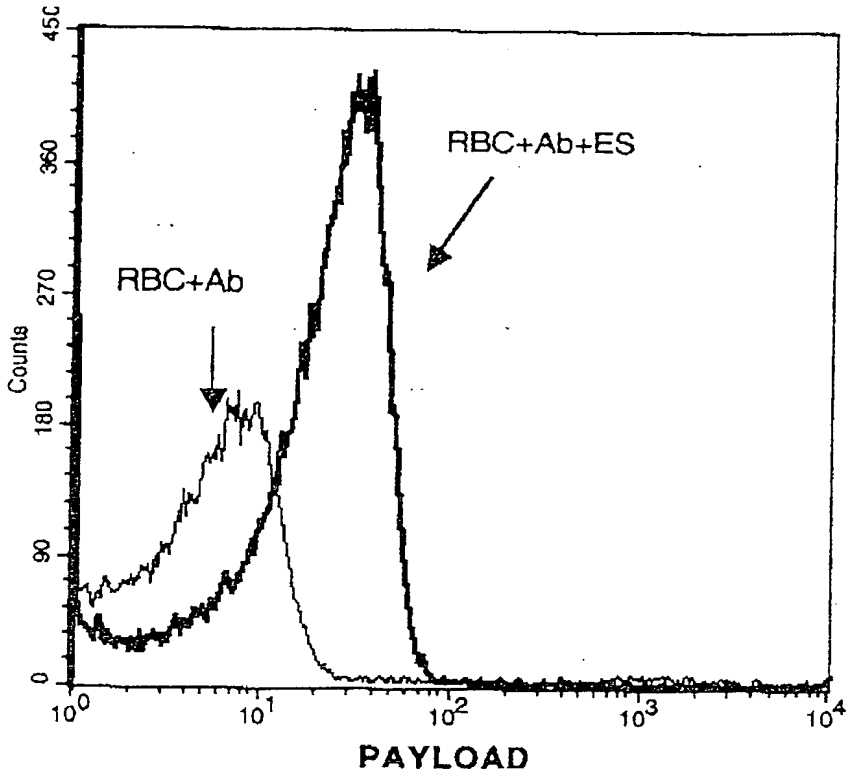


Fig. 1

A. ELECTROLOADING (EXPONENTIAL) OF ANTIBODY



B. ELECTROLOADING (SQUARE WAVE) OF ANTIBODY

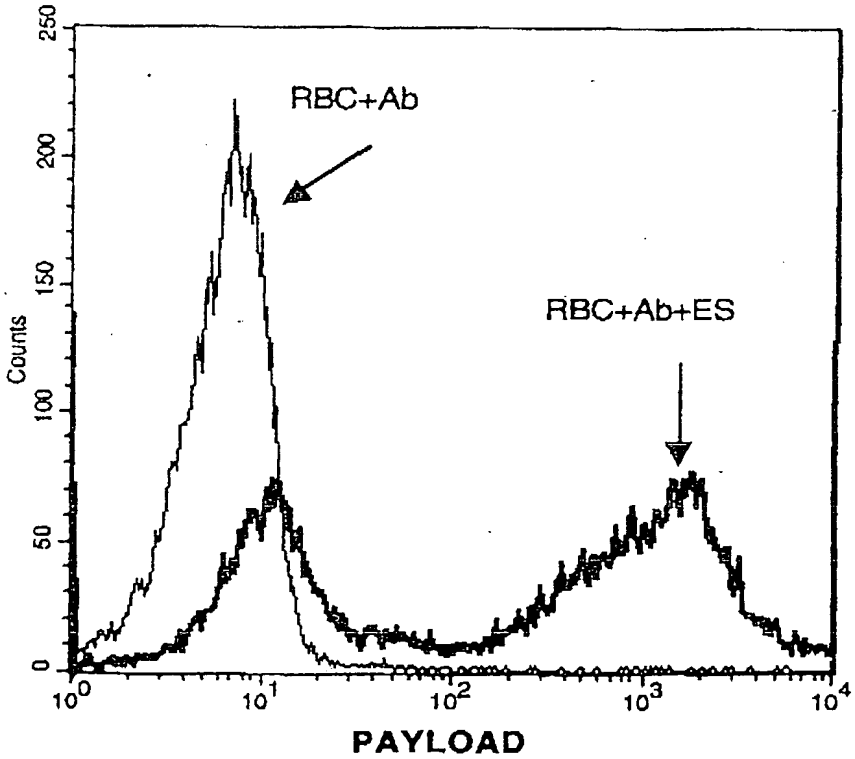


Fig. 2

COMPARISON OF OSMOTIC DIALYSIS LOADING PROTOCOLS PUBLISHED VS GENDEL TECHNOLOGY

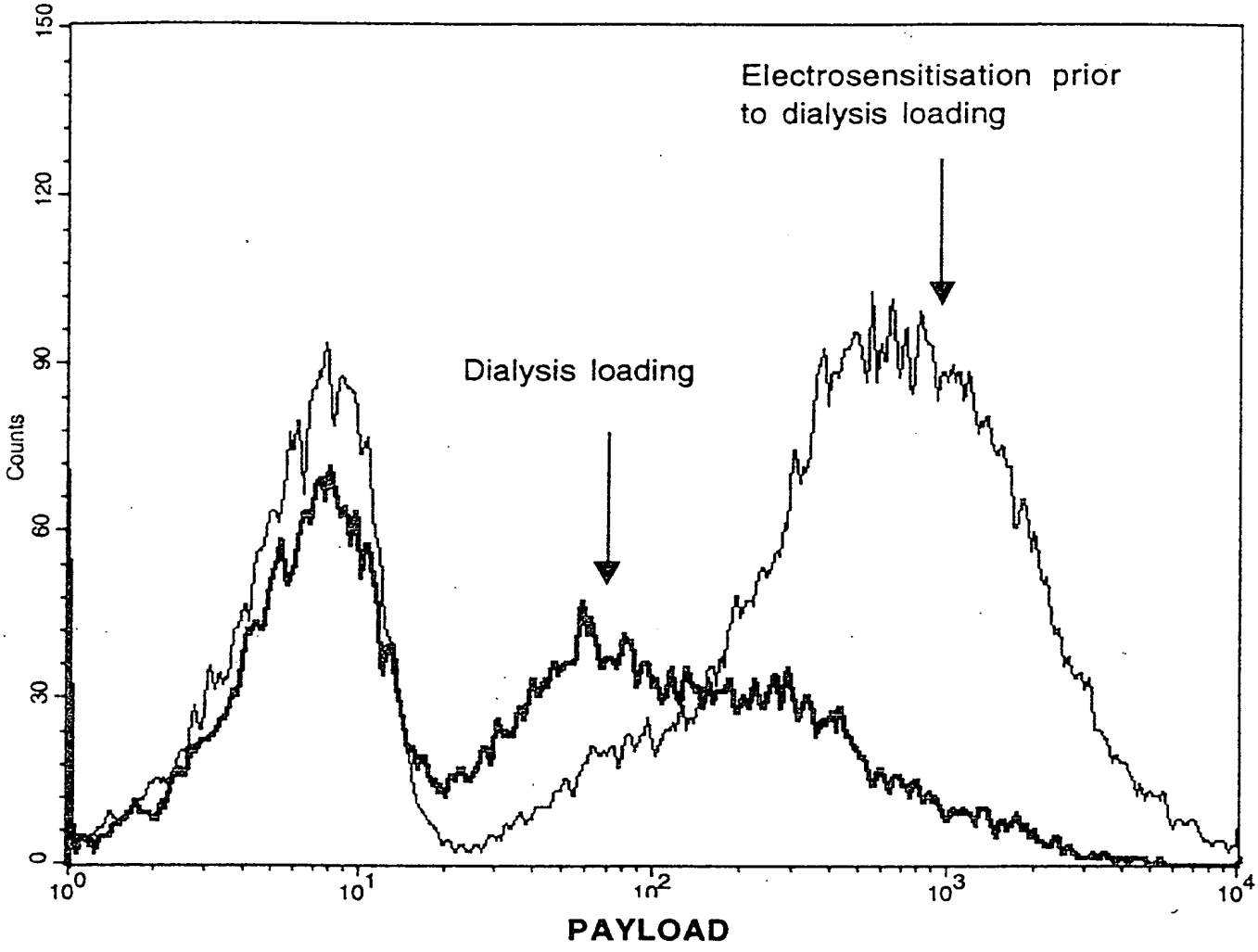
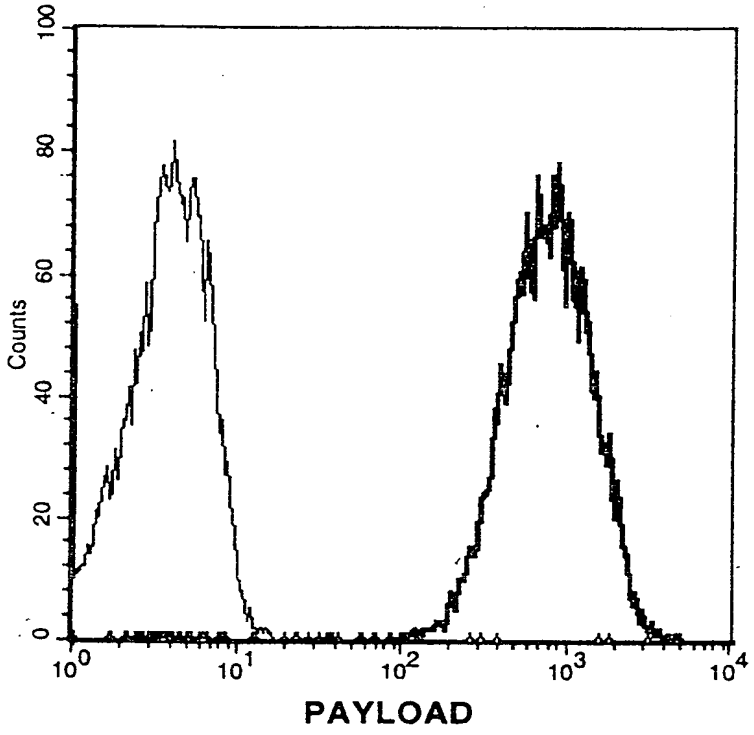


Fig. 3

A. GENDEL OSMOTIC LOADING WITH BUFFER A



B. GENDEL OSMOTIC LOADING WITH BUFFER B

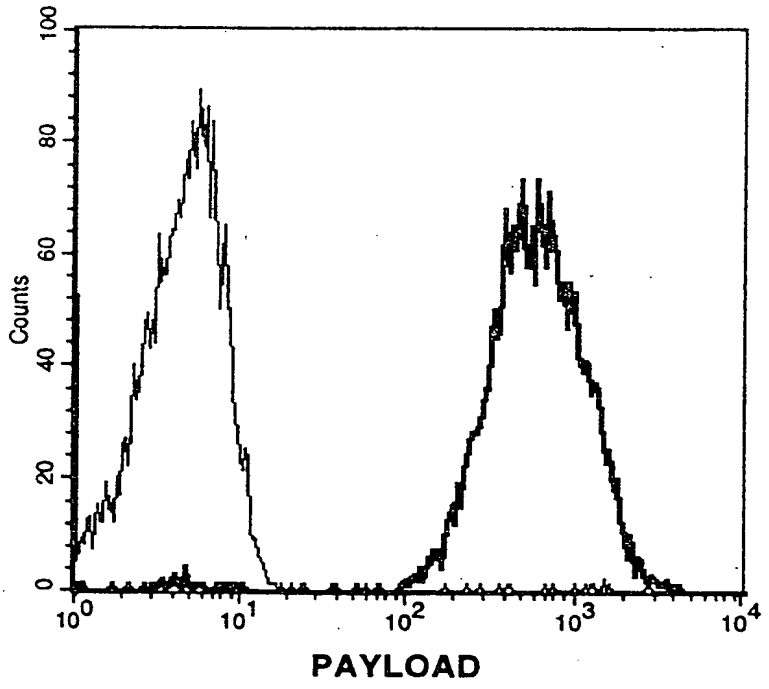
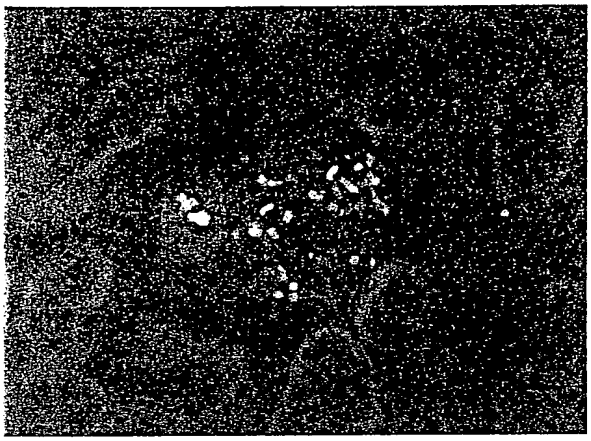


Fig. 4

A ULTRASOUND TREATMENT



B. NO ULTRASOUND TREATMENT

Fig. 5

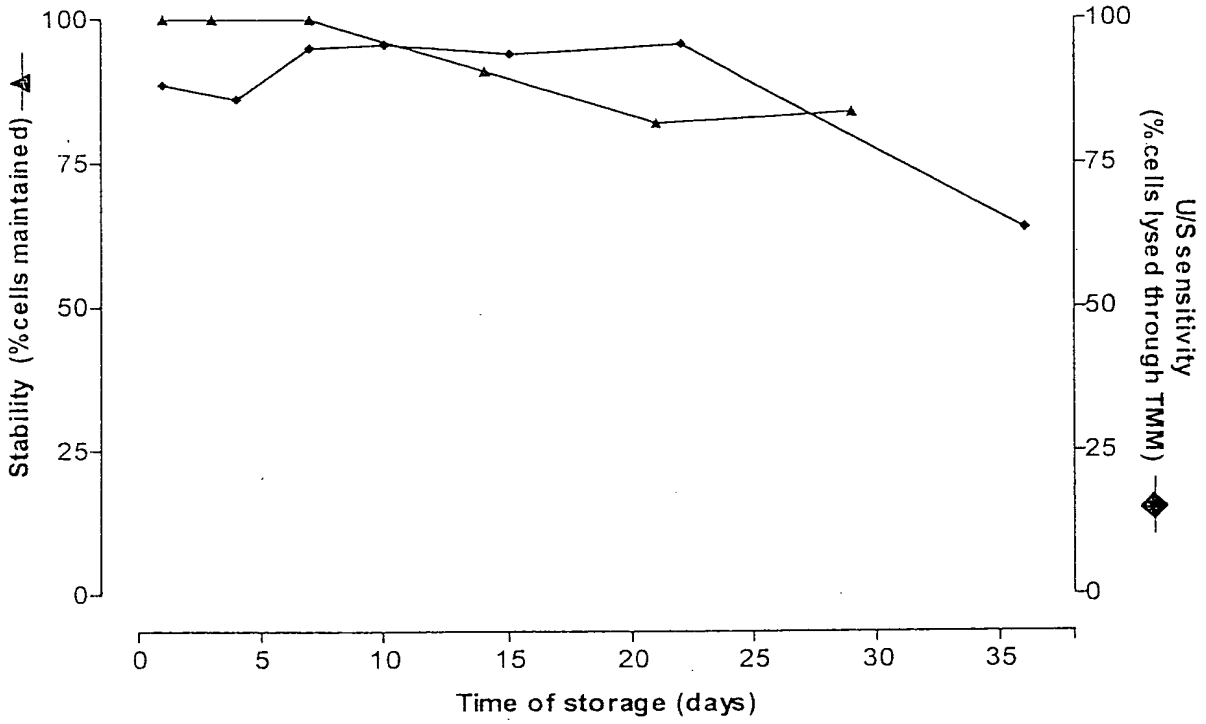

100
75
50
25
0
Stability (%cells maintained)
100
75
50
25
0
U/S sensitivity
(% cells lysed through TMM)
0
5
10
15
20
25
30
35
Time of storage (days)

Fig. 6

RETENTION OF PAYLOAD OVER 30 DAYS

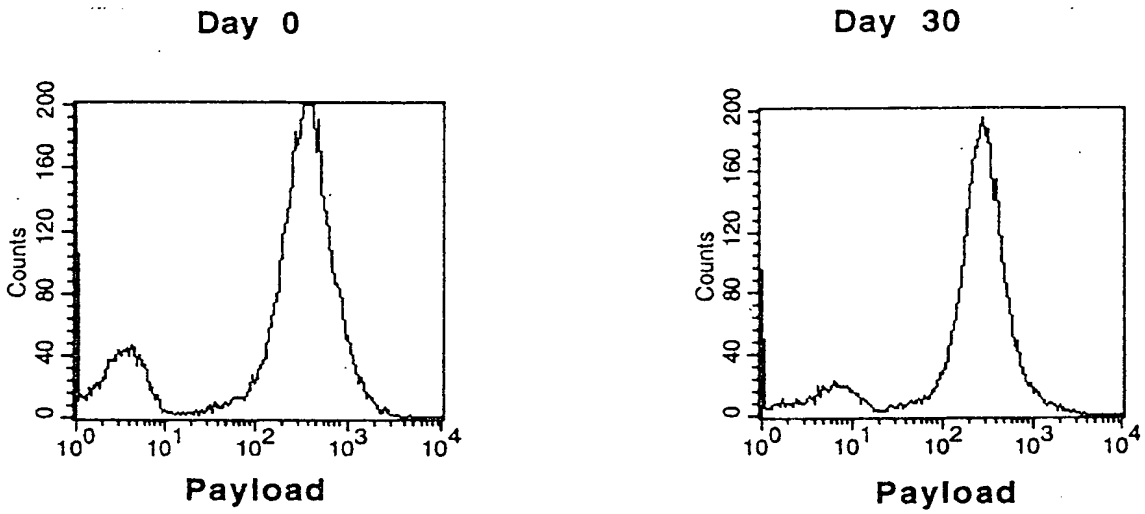


Fig. 7

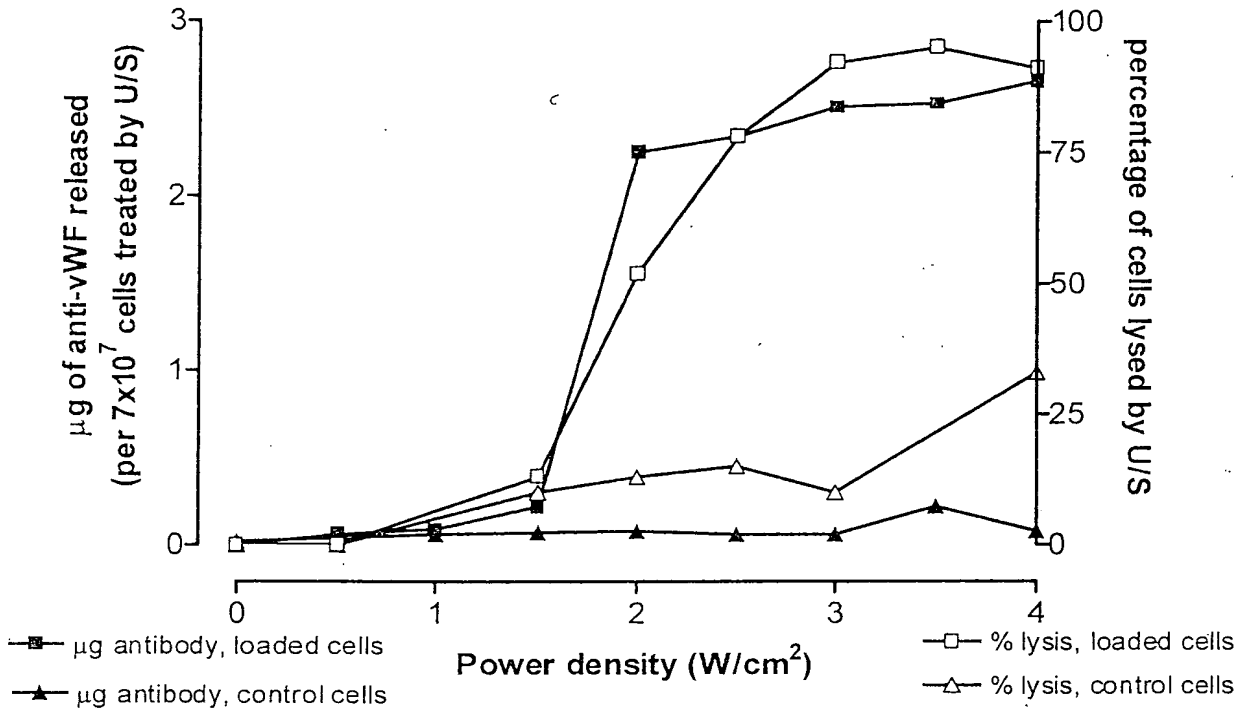

μg of anti-vWF released
(per 7x10^7 cells treated by U/S)
percentage of cells lysed by U/S
Power density (W/cm^2)
μg antibody, loaded cells
μg antibody, control cells
% lysis, loaded cells
% lysis, control cells
0
1
2
3
4
25
50
75
100

Fig. 8

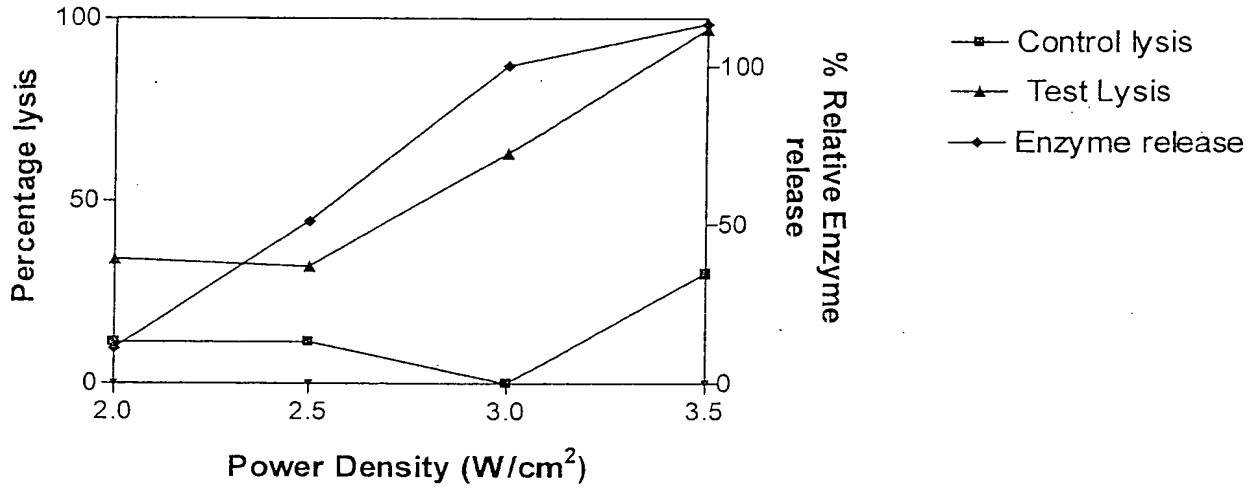

100
50
0
Percentage lysis
2.0
2.5
3.0
3.5
Power Density (W/cm²)
100
50
0
% Relative Enzyme release
Control lysis
Test Lysis
Enzyme release

FIG. 9

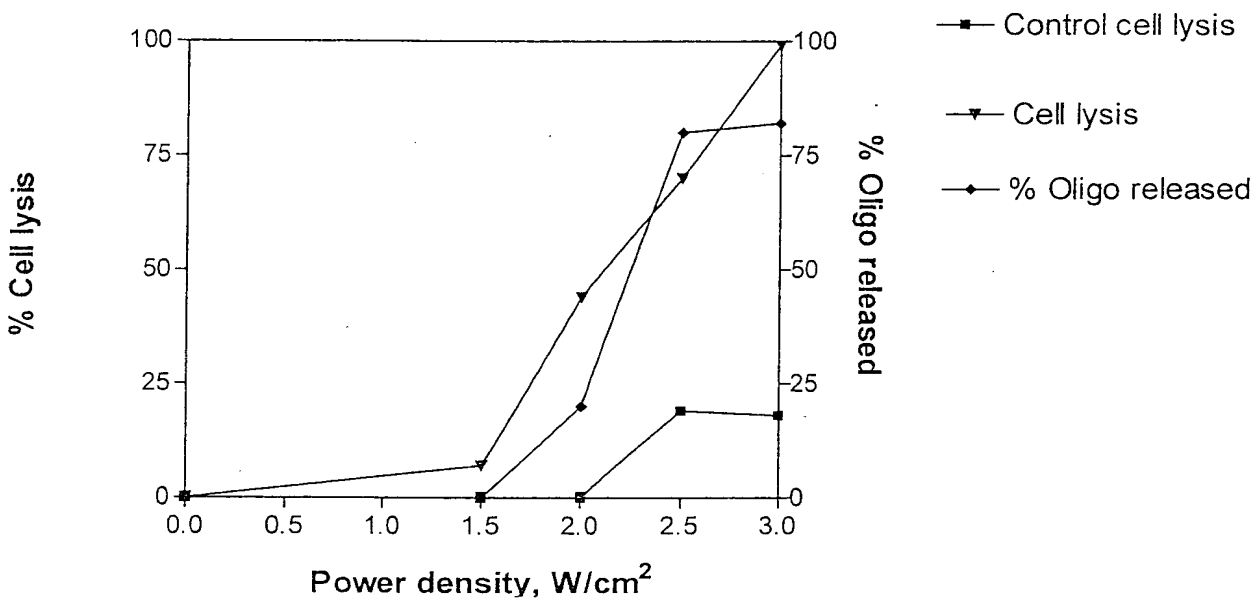

Control cell lysis
Cell lysis
% Oligo released
% Cell lysis
% Oligo released
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Power density, W/cm2

Fig. 10

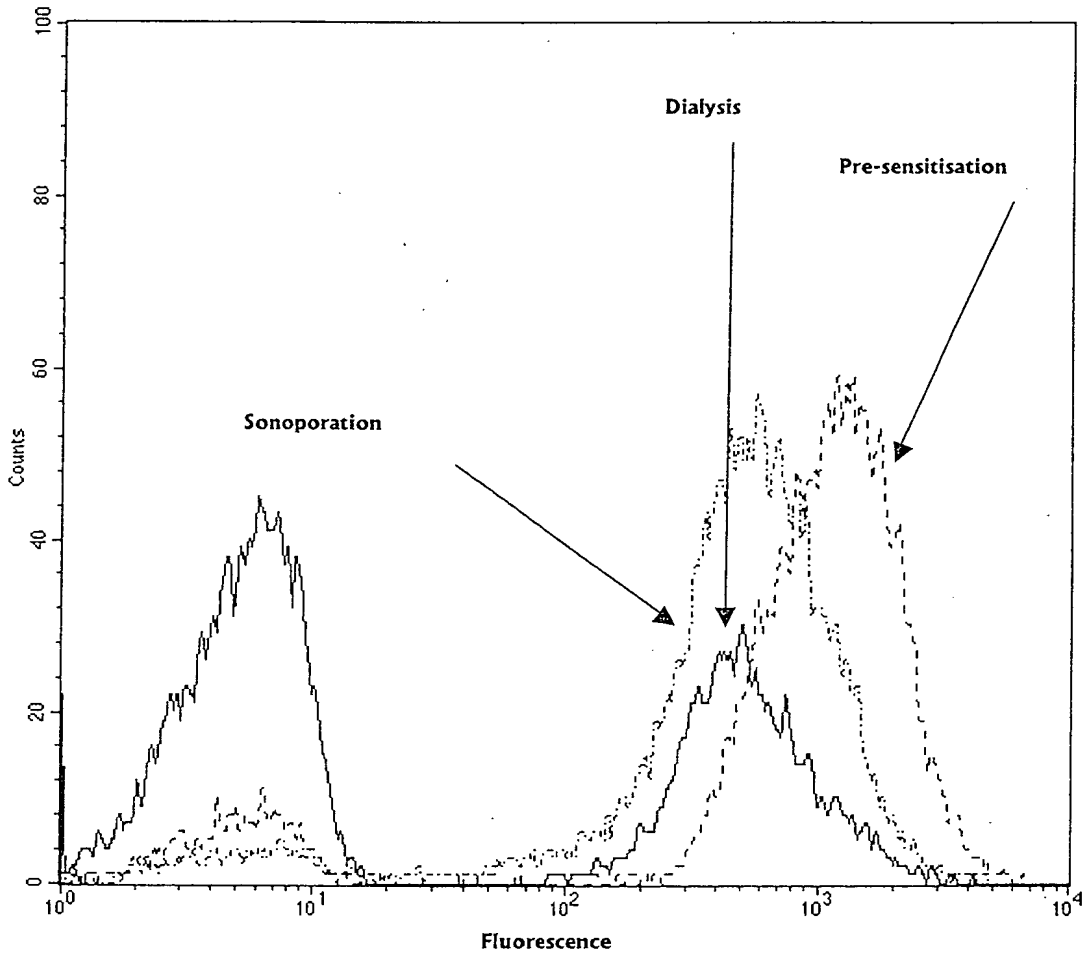

Dialysis
Pre-sensitisation
Sonoporation
Counts
Fluorescence
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4

Fig.11

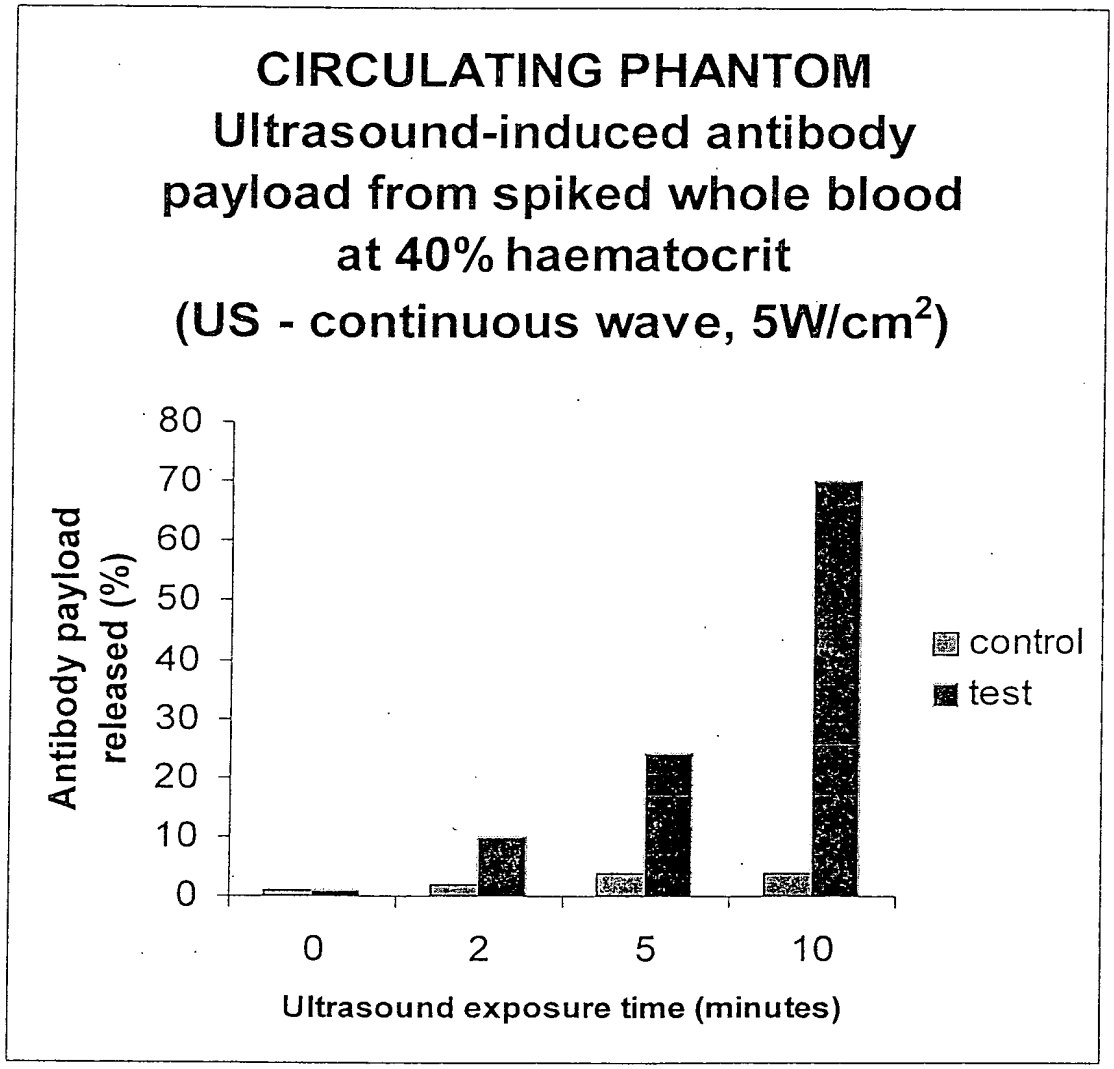

CIRCULATING PHANTOM
Ultrasound-induced antibody payload from spiked whole blood at 40% haematocrit
(US - continuous wave, 5W/cm2)
Antibody payload released (%)
80
70
60
50
40
30
20
10
0
0
2
5
10
Ultrasound exposure time (minutes)
control
test

Fig. 12